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BIOLOGICAL BULLETIN

TRICHODINA STEINII (C. AND L.) FROM *PLANARIA POLYCHORA* (O. SCHM.).

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In the spring of 1924 the junior author secured specimens of *Planaria polychora* O. Schm. from a pool several hundred yards west of the Biological Laboratory of the University of Virginia. The *Planaria*, in some cases, carried upon their surfaces specimens of *Trichodina Steinii*. These specimens afforded us an excellent opportunity to study the habits and anatomy of this species of large ectozoic peritrichous ciliate. Our observations led us to recognize two interesting facts concerning this species first described by Claparede and Lachmann (58).

The genus *Trichodina* is so closely related to *Vorticella* that it might be placed in the subfamily Vorticellidæ. Indeed Fulton (23) said of the subfamily Urceolariidæ, to which Minchin assigned *Trichodina*, "It is a matter of personal preference whether one considers the Urceolariidæ a subfamily of the Vorticellidæ . . . or as a separate family." With this close systematic relationship in mind, certain homologies between the anatomical details of *Trichodina* and those of *Vorticella* are of interest. The peristome and nucleus complex are clearly the homologues of the peristome and nucleus complex of *Vorticella*. In the free swimming *Trichodina Steinii*, the peristome remains closed (Fig. 3) in a manner that causes it to greatly resemble the closed peristome of a contracted *Vorticella*. A free swimming *Vorticella* has a posterior zone of cilia (Fig. 4). The homologue of these adoral cilia become fused at their bases to form the "velum" of *Trichodina*. *Trichodina Steinii*, in correlation with its habit as an ectozoön, has developed a horny flexible ring

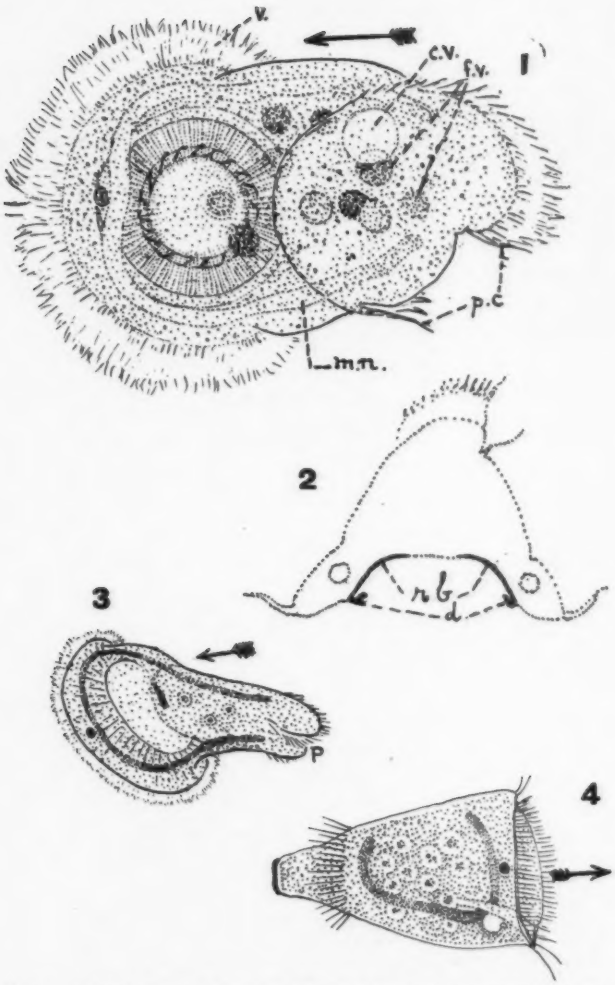

FIG. 1. Dorsal aspect of specimen as seen when fixed to or creeping over host. *v*, velum; *c.v.*, contractile vacuole; *f.v.*, food vacuoles; *p.c.*, cilia of peristome; *m.n.*, meganucleus (U-shaped). Observe the small micronucleus lying at outer margin of the U-shaped contour of meganucleus. × 1000.

FIG. 2. Transverse optical section through diameter of striated horny ring or ring band of adhesive organ. *r.b.*, horny ring or striated ring band; *d*, denticles or hooks. × 1000.

FIG. 3. Free swimming *Trichodina Steinii*. *p*, peristome closed. Note position of micronucleus and meganucleus with reference to the animal's swimming posteriorly, as indicated by the arrow.

FIG. 4. Free swimming form of *Vorticella*. Note position of micronucleus and meganucleus with reference to the animal's swimming anteriorly, as indicated by arrow.

(ring band) as a conspicuous feature of its "adhesive organ." In this complex adhesive organ, with its velum, Louis Agassiz (50) saw homologies between it and the details of an hydromedusa. Because of this he looked upon the *Trichodina* as being the medusa of *Hydra*. Attempting to establish analogies may, therefore, lead one into strange ventures. But a further homology may be seen in the horny ring of the adhesive organ of *Trichodina Steinii*. This ring is of a chitinous texture and lies at the posterior extremity of the body of *Trichodina*. Fauré-Fremiet (05) studied the adhesive substance that *Vorticella* elaborates when it fixes itself to a surface. He found that this adhesive material was a "sécrétion chitineuse." If now the reader will look at our figures 3 and 4, he will see that in the free swimming *Vorticella* and *Trichodina* at the posterior end of each of these ciliates is a region concerned with the secretion of a horny material. In *Vorticella* the horny substance is the adhesive material, whereas the homologue of this horny substance becomes the horny ring or "striated ring band" of the adhesive mechanism in *Trichodinii Steinii*.

Perhaps a more interesting comparison may be drawn between *Vorticella* and *Trichodina* than that based upon homologies. This contrast presents itself in the polarity of the two protozoa. *Vorticella* has its anterior-posterior polarity normal. When a *Vorticella* moves about it swims with its peristome forward as indicated by the arrow in Fig. 4. The careful studies of Jennings (04) indicate that it is a matter of great importance to an animal like *Paramœcium* that its peristome lies towards the line of travel. To reverse this attitude would appear, therefore, to be a radical change. This, however, is what has taken place in *Trichodina Steinii*. These ciliates become quite active when their host is placed under the compound microscope's light and creep about over the surface of the host freely. In all cases they advance posteriorly as they cling lightly to the surface of the host with their adhesive organs. If the disturbance be continued long enough, they will leave the host and swim off into the water. Even the free swimming specimens travel *posteriorly*. When thus traveling, they have the peristome closed and inactive while the velum and horny ring are expanded (Fig. 3). There is thus a complete reversal of polarity of this animal both when it creeps over a

surface and when it swims. This reversal of polarity has been overlooked by the earlier observers of *Trichodina Steinii*. Kent (81) implies that *Trichodina pediculus* (Ehr.) has the usual polarity. Fulton (1923) in making a thorough review of the literature of this genus and in his own observations, seems not to have encountered this reversal of physiological and morphological polarity. This author, as well as earlier investigators, has also overlooked the homologies that we have drawn between this ectozoic peritrich and *Vorticella*.

This animal should, therefore, serve as the basis of some interesting work done upon its physiological axial gradient. From what we have seen of its morphology we expect to find that its axial gradients have been reversed in their sequence; for it is seen that there has been a morphological reversal, despite the homologies that we have indicated. In *Vorticella* there is an emphasis placed upon the anterior region in that the loop of the meganucleus and the micronucleus lie in that region. In *Trichodina Steinii* the morphological emphasis is reversed; for in this form the loop of the meganucleus and the micronucleus lie in the posterior region of the cell.

Thus it appears that in *Trichodina* rather complex adaptive structures are but the homologues of simpler structures found in *Vorticella;* but that correlated with the ectozoic mode of living *Trichodina* has undergone a reversal of polarity that involves both its physiological axis and its nuclear complex.

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STUDIES IN ARTIFICIAL PARTHENOGENESIS.

V. The Anomalous Action of Mercuric Chloride.

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Traube[1] in 1909 made the claim that substances of low surface tension were more effective in producing parthenogenesis than those of higher surface tension. In 1913 and again in 1915, Heilbrunn[2] proposed a theory of membrane elevation in the sea-urchin egg, according to which the lifting off of the membrane (in this egg) is the direct result of a lowering of its surface tension. If this surface tension theory of membrane elevation is correct, only those substances which cause a decrease in surface tension can produce typical membrane elevation. So far as previous experiments have gone, this is true. There is no case in which membrane elevation has been produced by an agent which does not sharply lower surface tension (Heilbrunn, '24).

During the past summer an apparent exception was discovered. In the course of some work which we were doing with dilute solutions of mercuric chloride in sea-water, my assistant Mr. D. E. S. Brown called my attention to what was apparently true membrane elevation in the egg of the sea-urchin *Arbacia*. Beautiful wide membranes arose from the egg surface. That these were truly elevated membranes (not swollen membranes) was shown by the fact that they could be made to collapse on the addition of egg albumin to the sea-water.

The parthenogenetic action of mercuric chloride was known to F. R. Lillie and he mentions it briefly in a paper published in 1921[3] (see p. 140).

It seems certain that mercuric chloride as such could have no great effect on surface tension. Apparently, therefore, the fact that the mercury salt causes membrane elevation can not be made to fit in with the surface tension theory. Because of this apparent conflict, it was thought worth while to further investi-

gate the action of the mercuric chloride, with a view to the possible abandonment of the surface tension theory. Although many experiments were performed, only a few of them will be considered and these rather hastily. They suffice to show that the action of mercuric chloride is not a simple one.

If an *m*/10 solution of mercuric chloride is diluted a thousand times with sea-water, then an "*m*/10,000 solution of mercuric chloride in sea-water" is obtained. A solution of this sort is very favorable to membrane elevation. Solutions decidedly more concentrated than *m*/10,000 do not act as well. In an *m*/1000 solution, the membranes, if they lift off at all, become only partially elevated, apparently they stick to several points of the egg surface as they attempt to lift away from it. On the other hand extreme dilutions are also ineffective, although it is possible to obtain good membrane elevation in solutions as weak as *m*/100,000. Thus there is a wide range of concentration in which the mercuric chloride is effective. Most of the experiments were made with an *m*/10,000 solution (in sea-water).

On exposure to the mercuric chloride, membrane elevation does not occur immediately. After 3 or 4 or 5 minutes, the membranes can be seen rising from the surface of the eggs. The effect of the sublimate is not solely a cortical effect. After eggs have been exposed for about 12 minutes other changes may begin to appear. In many cases the cell undergoes what is apparently a cell division. At any rate it becomes constricted. Usually this constriction divides the egg unequally, a large cell and a small cell are formed. In such cases of unequal division the smaller cell usually contains a mass of pigment and the larger cell is comparatively colorless. Now and then instead of an unequal division there is a furrow around the very center of the egg with an accumulation of pigment granules underneath the furrow. No attempt was made to discover whether nuclear changes accompanied the changes in the cytoplasm. If the constrictions of the cytoplasm do represent anything at all comparable to a normal cell division, it is surprising that they occur so soon after the eggs are exposed to the mercuric chloride. Typically the Arbacia egg does not divide until 50 or 60 minutes have elapsed after fertilization. The cytoplasmic constrictions

just described make their appearance 12 or 15 minutes after the eggs are placed in the mercury solution. The constriction of the cytoplasm is not followed by anything approaching normal development, even if the egg is removed from the solution of mercuric chloride and placed in normal sea-water.

Ordinarily when Arbacia eggs are exposed to an *m*/10,000 solution of mercuric chloride in sea-water, only about 10 per cent. of the eggs undergo membrane elevation. Why 10 per cent. of the eggs should throw off perfect membranes and the other 90 per cent. should show no change at all, was for a time a mystery.

After a number of experiments under diverse conditions, it was found that the percentage of eggs undergoing membrane elevation could be greatly increased by allowing the eggs to age. If eggs are taken from the ovary and placed directly into dilute solutions of mercuric chloride, no membrane elevation occurs. On the other hand if they are allowed to lie in sea-water in shallow dishes for 5 or 6 hours, practically a hundred per cent. will undergo membrane elevation when exposed to the mercuric chloride. In working with this reagent it is therefore advisable to allow eggs to age before starting to experiment with them.

The effect of aging is shown in the following table. The first column of this table gives the number of minutes the eggs were allowed to remain in sea-water before they were treated with *m*/10,000 mercuric chloride. Only the eggs of a single female were used but these were very plentiful. On removal from the ovary they were placed in 50 cc. of sea-water in a fingerbowl. After a time, as a result of the constant removal of material, the eggs at the bottom of the fingerbowl were no longer evenly distributed, some being more closely clustered than others. In the denser masses of eggs, the aging process apparently proceeded at a slower rate and this accounts for the irregularities in the table. The second column shows the percentage of eggs undergoing membrane elevation when subjected to mercuric chloride. To obtain this percentage two hundred eggs were counted in every instance except in the case of the eggs aged for 268 minutes. Only a hundred of these were counted.

Time Eggs Remained in Sea-water before Treatment with $HgCl_2$, Minutes.	Percentage of Eggs Showing Membrane Elevation.
0	0
5	6½
10	7½
15	10
20	10
30	11½
40	13
50	14½
60	17
81	17½
103	17½
128	15½
158	16
193	18½
238	30½
268	21
300	62½
345	94½

Although the table is not as regular as might be wished, it is clear that as the eggs lie in sea-water they become increasingly susceptible to mercuric chloride. This fact is interesting for at least two reasons. In the first place it apparently does not harmonize very well with the fertilizin theory of fertilization as developed by F. R. Lillie.[3] Aging of eggs, according to this theory, involves a loss of fertilizin, a material which is regarded as essential to fertilization, and yet in the experiment described above, such aging favored a cortical change characteristic of fertilization. But the case of mercuric chloride is certainly a special one, and it is doubtful if any general conclusions for or against the fertilizin theory can be derived from it. This will be more obvious from the later discussion.

Our main interest is elsewhere. Why do eggs after standing in sea-water respond more readily to treatment with mercuric chloride? Possibly in or around eggs fresh from the ovary there is some substance present which exerts an interference. This substance escapes on standing; it may be gaseous. On a priori grounds one would suspect carbon dioxide.

Experimental evidence favors this conjecture. When eggs

were allowed to stand until they became highly susceptible to mercuric chloride and were then treated with a solution of the sublimate in sea-water which contained a trace of hydrochloric acid, they did not lift off membranes. If now the solution of mercuric chloride in acidified sea-water was shaken vigorously for a few minutes it again became potent as a stimulant to membrane elevation. In one experiment 4 cc. of *n*/10 HCl was added to 200 cc. of sea-water. To this was added 11 cc. of *m*/1000 $HgCl_2$. The resultant solution had a p_H below 6.8 and caused no membrane elevation. It was shaken until the p_H rose to above 7, but it still remained ineffective. It was then shaken vigorously for several minutes, and the p_H rose well above 7. After such shaking it produced membrane elevation generally. The effect of the shaking is to remove carbon dioxide from the solution. It seems certain from this experiment that the presence of carbon dioxide interferes with the production of membrane elevation by mercuric chloride.

Our first point then is the fact that mercuric chloride causes membrane elevation only in the absence of any considerable quantity of carbon dioxide.

A second point, which was not investigated as closely as it might have been, is the fact that sublimate treated eggs, if centrifuged one or two minutes after the exposure begins, do not lift off membranes. In one experiment no membranes were lifted off when eggs were centrifuged either one or two minutes after they had been placed in a *m*/10,000 $HgCl_2$ solution. Those centrifuged 3½ minutes after being placed in the solution showed membrane elevation in 91 out of 100 cases. The centrifugal force used was 4,968 times gravity and in this experiment the eggs were centrifuged for 40 seconds in each instance. Apparently it is not necessary to centrifuge the eggs for as long a time as 40 seconds. In other experiments it was found that centrifugal treatment for only 5 seconds was enough to prevent all but a small percentage of the eggs from undergoing membrane elevation. The reason for the effect of the centrifuge is not certain. A plausible hypothesis, but by no means the only one, is that the mercuric chloride reacts with the jelly of the egg to form mercurous chloride and chlorine, and that it is the chlorine which is

important for membrane elevation. Centrifuging removes the jelly from proximity to the egg surface. In favor of this view is the fact that mercuric chloride regularly does break down into calomel (or mercuric oxychloride) and chlorine in the presence of organic substances (Mellor,[4] Gmelin-Kraut [5]). On the other hand it should be pointed out that if the jelly is removed from the eggs either by shaking or centrifuging or both, and the eggs are then subjected to mercuric chloride solution, they undergo membrane elevation just as well as when the jelly is present. This fact need not interfere with our hypothesis. We can assume that although ordinarily the mercuric chloride reacts with the jelly to form chlorine, in the absence of chlorine it may react with the cortex of the egg itself.

A test of this point might be made by removing the jelly, treating with mercuric chloride and then centrifuging. If the above interpretation is correct then centrifuging of jelly-free eggs should not interfere with membrane elevation produced by the mercury salt. Unfortunately such a test was not made.

But there is other evidence in favor of the view that mercuric chloride reacts with some part of the egg. Sublimate solutions lose their effectiveness when allowed to stand over eggs. In one instance, after an m/10,000 solution had stood over eggs for 10 hours it in large measure lost its effectiveness as a stimulant to membrane elevation. Although a control of the same solution produced typical membrane elevation, the solution which had stood over eggs caused at best only a partial and slight membrane elevation. Even in the absence of eggs an m/10,000 mercuric chloride solution slowly loses its power to cause membrane elevation. The loss of this power is much more rapid in the presence of eggs.

If we assume that mercuric chloride gives off chlorine and it is this substance which causes membrane elevation, it should be possible to duplicate the action of the mercury salt with chlorine itself.

Chlorine gas was manufactured by adding hydrochloric acid to potassium permanganate and the gas was then passed through sea-water. If two drops of the solution of chlorine in sea-water are added to 20 cc. of sea-water the resultant solution is then

effective in inducing membrane elevation. The results with chlorine itself are not as good as those with mercuric chloride. It is hard to regulate the amount of chlorine and only a very narrow range of concentrations may be used. One drop more or less of chlorine water added to 20 cc. of sea-water would often determine whether the resultant solution would be effective or not. Moreover the number of eggs introduced into the chlorine solution was an important factor. Thus in one experiment two stender dishes were each filled with 20 cc. of sea-water plus 2 drops of chlorine water. Many eggs were placed in one of the dishes, only a few in the other. In the latter case 78 per cent. of the eggs underwent partial or complete membrane elevation; in the dish containing many eggs no membrane elevation occurred at all.

It is easy to suppose that the addition of mercuric chloride to eggs furnishes a mechanism for supplying approximately the proper dose of chlorine to the egg surface. This is probably the reason that solutions of chlorine alone are not as effective as the mercuric chloride.

An argument that might be advanced against the idea that the action of mercuric chloride is due to chlorine is the fact that mercuric nitrate is also effective in causing membrane elevation. In order to exclude the presence of chlorine, the mercuric nitrate was made up in a 0.9 *m* sugar solution. A solution roughly *m*/10,000 was prepared. The action of such a solution is very peculiar. Extremely wide membranes are lifted off, membranes which are quite different in appearance from the normal-looking ones produced by mercuric chloride. It would be easy to show that a reaction occurs between the sugar and the mercuric nitrate, but no effort was made to determine the products of such a reaction. However it is believed that one of the reaction products causes the membrane elevation.

From the evidence cited it is obvious that the action of mercuric chloride is complex. It appears certain that the reagent does not act in its original form but undergoes some sort of a chemical transformation. There is good reason to believe that chlorine is produced, and that this substance acts on the eggs and incites membrane elevation.

If this view is correct then the fact that mercuric chloride solutions are effective in causing membrane elevation can not be used as an argument against the surface tension theory. If the sublimate forms chlorine we know that a substance of extremely low surface tension must be present.

It is possible that many more facts with regard to the action of mercuric chloride on egg cells or cells in general might easily be obtained by a further study of the sea-urchin egg in the presence of this reagent. Our only interest in the subject lay in the possibility of overthrowing the surface tension theory of membrane elevation. As soon as it was evident that no such overthrow was possible on the basis of the mercury evidence, no additional experiments were planned.

Summary.

1. Dilute solutions of mercuric chloride in sea-water cause typical membrane elevation in the sea-urchin egg in spite of the fact that they presumably do not lower surface tension.

2. The action of the mercuric chloride is favored by aging the eggs. Eggs fresh from the ovary are not acted upon, and the percentage of membrane elevation on treatment with the sublimate solution increases in proportion to the time the eggs have stood in sea-water before being subjected to the reagent.

3. The favorable effect of aging is apparently due to the removal of carbon dioxide. The addition of carbon dioxide prevents membrane elevation by mercuric chloride.

4. If eggs are centrifuged one or two minutes after the treatment with mercuric chloride is begun, membrane elevation is generally prevented.

5. Solutions of mercuric chloride in contact with eggs lose their power of provoking membrane elevation.

6. The facts cited in 4 and 5 are regarded as evidence in favor of the view that mercuric chloride reacts with the jelly or cortex of the egg to form chlorine. Such a reaction is in accord with the usual behavior of mercuric chloride in the presence of organic materials.

7. Chlorine gas is effective in producing membrane elevation.

8. The action of mercuric chloride in causing membrane eleva-

tion is probably due to the formation of chlorine. Since chlorine has a very low surface tension, the fact that mercuric chloride causes membrane elevation can not be used as an argument against the surface tension theory.

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THE SPERMATHECA OF *EURYCEA BISLINEATA*.[1]

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Since 1785 it has been known, first through Spallanzani, that fertilization in salamanders is internal. Later there were found at intervals in several species "receptacula seminis" into which spermatozoa are received and stored. So far as is known in most species of salamanders the spermatozoa are transferred some time before egg-laying to the cloaca of the female by means of spermatophores deposited by the male and received by the female either actively or passively, the method of deposition and reception varying somewhat in the few species which have been noted at breeding.

The "receptacula seminis" or spermathecæ[2] of several forms has been noted by Rathke (1820), Leydig ('53), Siebold ('58), Jordan, Fisher and Stieda ('91), and Kingsbury ('95). The work of Dr. Kingsbury is especially important as a comparative study of the spermathecæ of *Necturus maculatus*, *Diemyctylus viridescens*, *Desmognathus fuscus*, *Amblystoma punctatum*, *Plethodon glutinosus* and *Spelerpes bislineatus* (*Eurycea bislineata*). In all of these forms the spermatheca is dorsal to the cloaca and consists of a number of tubules. These tubules open severally into the dorsal wall in *Necturus*, *Diemyctylus*, and *Ambystoma* and cover comparatively large areas in this region. In *Desmognathus*, *Plethodon* and *Eurycea* the organ is more compact and opens by a single central tubule into the cloaca.

Kingsbury describes but one specimen of *Eurycea bislineata* taken in October and containing no spermatozoa. It is the purpose of this paper to continue the work on *Eurycea*, describing the mature spermatheca at various seasons and in the course of its development.

[1] Contributions from the department of Zoölogy, Smith College, No. 131.

[2] Dr. H. H. Wilder calls attention to the fact that the correct word is Spermatotheca, using the root of the word (spermato-) instead of the nominative stem.

I.

The Mature Spermatheca.

The spermatheca of *Eurycea bislineata* is a compact, definitely bounded organ. Lying in a median dorsal position with reference to the cloaca it extends from the opening of the organ, a point cephalad to the vent of the cloaca, to the posterior wall which lies above the vent of the cloaca. A mature spermatheca is usually from 1/2 mm. to 2/3 mm. in antero-posterior extent, approximately as wide as it is long and slightly higher, dorso-ventrally, than it is wide—that is, somewhat conical in shape.

The spermatheca is a sac of tubules. The storage tubules, where the spermatozoa lie in season, are flask-shaped, from seven or eight to sixteen in number, and they converge into one central tubule which bends abruptly and opens into the cloaca. The sac is of heavily pigmented connective tissue. In the posterior wall and the floor of the sac this is thickest, most coarse and black, and the flask ends of the tubules are imbedded in the mass as in sockets. The coarse, pigmented tissue thins out in the anterior region and there is none of it in proximity to the necks of the flask-shaped tubules or the central tubule.

The extreme caudal end of the sac is but loosely connected with the cloacal wall and surrounding tissues, being held in place by scattered coarse strands of connective tissue. But approaching the mouth of the organ, where the pigmented floor thins out, it becomes more a part of the cloacal structure, the smooth musculature of the cloacal walls merging with the similar fibers of the interior of the spermatheca.

The arrangement of the tubules is noteworthy. The flask-shaped ends lie in the floor of the sac imbedded in pigmented tissue. Some are quite anterior, being anterior to the plane of the opening of the central tubule into the cloaca. But these most anterior tubules, one on each side of the median line, have their sockets of pigmented tissue though pigment is otherwise sparse in this region. The necks of the flasks converge in the posterior end of the sac and form the central tubule which runs dorsally anterior. Thus some of the flasks have very long and narrow necks. The convergence of the necks occasions a large reservoir which narrows into the central tubule proper.

The lining of the whole system of tubules is of most delicate columnar epithelium which extends into the cloaca in the region around the mouth of the central tubule. It is a markedly different tissue from the mucous lining of the cloaca. There is no mucous secretion from the spermatheca; nor at times is there any secretion of any kind as far as can be ascertained by staining reactions. Save for the spermatozoa during the breeding season the lumina may be clean and clear. Some series, however, show a faint indefinite substance, rather stringy, in the flask ends. The necks and central tubule may show slight traces of the matter also, though it is rare in the central tubule which is generally unusually clear.

There is a slight differentiation in the lining tissue in different parts of the system. In the blind ends the cells are long. The bases are densely granular with large nuclei. The apices stain faintly; they seem filled with minute, transparent globules. The apices are irregular—they do not form an even border for the lumen. They give a picket-fence appearance. In some series these inner halves of the cells are shrunken so that their ends appear as amœba-like processes, or as if they were in an exhausted state. Yet these conditions occur during the fall and winter months during which it is to be supposed that there is no reason for activity of the cells as no spermatozoa are present.

The cells of the necks of the flask tubules are shorter than those of the blind ends and produce a much more even border—that is, there is no appearance of shrinkage of the cells. The apices are rounding. It is in the central tubule, however, from the region of the convergence of the necks to the area surrounding the mouth of the cloaca that there is the most marked difference from the lining of the flask ends. The appearance of the central duct is constant at all seasons. These cells do not differ as to bases but the apices are very slightly longer and stain even more delicately than do those of the flask ends—there is no globular appearance. The apices fit perfectly together and form a smooth, even surface for the lumen. No variation of this condition has been noted in any mature spermatheca.

The size of the lumen of the central tubule varies—it is a reservoir at the convergence of the necks. In what are presumed

to be very old animals there are two of these reservoirs formed by the necks of each side; these join, narrow and become the central tubule. The lumen of this tubule near the opening into the cloaca is often very small—about 1/100 mm.—but it may be as wide as 4/100 mm. The lumina of the necks vary around 1/100 mm. and those of the flask ends are usually about 6/100 mm. in diameter.

The dense coat of pigmented tissue, covering and binding in place the tubules, has been spoken of. This coat is thin, sometimes of but a few strands on the roof of the spermatheca. It is along this dorsal region that the central duct lies. This duct and the necks are surrounded by massed smooth muscle fibers which do not differ from the smooth muscle of the walls of the cloaca.

Unless injected, it is difficult in amphibian material to study the blood supply of an organ. Red blood corpuscles stain vividly with eosin and when present in numbers indicate blood vessels and capillaries even when dense pigment is present. In one series of a spermatheca which is on the verge of maturity many blood corpuscles have remained in this region. It is evident that the blood supply is rich. Corpuscles running single file everywhere throughout the region of the spermatheca indicate a close network of capillaries and some larger blood vessels are present. Only injection would show certainly, however, just where these vessels branch from the dorsal aorta which lies immediately above the spermatheca.

The mating of *Eurycea* takes place in the spring. There have been no spermatozoa found in the spermatheca during the fall and winter months. April may be taken as the average mating season though there must be as variable a season for mating as there is for egg-laying and many early and late dates have been recorded for *Eurycea* eggs.

Spermatozoa are found in the flask tubules usually in dense, orderly whorls similar to the groups found in the vas deferens of the male. They may be, however, scattered and tangled. No spermatozoa have been found in the central tubule in any series I have sectioned and in but one are there any in the necks of the tubules. This is a sagittal series and some sections,

in one side of the spermatheca, pass longitudinally through a flask and its neck. The spermatozoa are streaming in a mass from the flask into the neck. But other sections show that they have not proceeded as far as the central duct.

Flask tubules containing spermatozoa show a modification of the epithelium. The tall columnar cells are unrecognizable. There is instead a narrow row of flat cells utterly different in appearance from columnar cells. The delicate part of the cells has completely disappeared and no ragged nor shrivelled edges even indicate its former presence. The width of these cells is 1/100 mm. in comparison with measurements of 3/100 and 4/100 mm. during the non-breeding periods.

Some of this difference might be accounted for by presuming that the tubules are stretched by the mass of spermatozoa contained within them. However, there does not seem to be any such stretching. Measurements of the diameter of the tubules during the breeding season are normal corresponding to the size of the spermatheca. Also when the flasks are cut squarely through, the whorl of spermatozoa is shown lying in the lumen with no adherence to the walls. There is no evidence of crowding or packing.

II.

Development.

The above description is of a typical mature spermatheca following at least one season of egg-laying. The organ in some specimens which are larger and presumably older differs in some details and will be discussed at the end of this part on the development.

The development of the spermatheca is to be considered with reference to the growth of the cloacal region also. There is a great deal of change in the whole region from the time of metamorphosis to sexual maturity.

The youngest specimen prepared was a 45 mm. female identified by Mrs. Wilder as a stage in early metamorphosis. There is no indication of any spermathecal tubules though the beginning of some cloacal glands may be apparent. In this paper the term "cloacal glands" is applied to the mucous glands of the

cloaca. Kingsbury speaks of "ventral glands" but since many of the tubules are in the lateral walls of the cloaca and far dorsal, some beginning near the walls of the spermatheca, the more general term "cloacal glands" may be accurate enough. The cloaca at this stage is very simple; the walls are smooth and the only projection or fold is a small median papilla.

Other young animals prepared are 58 mm., 59 mm., 64 mm., 66 mm. and 68 mm. total length. But after metamorphosis there is no definite guide to the age of the animals as length is not a criterion and the size of the gonads is variable in all ages at various seasons, and within this range of lengths mature organs are found. A 67 mm. female killed in April was functional with spermatozoa in the flask tubules. A 68 mm. animal killed in June had a mature spermatheca which undoubtedly had been functional at least during the recent breeding season. Another April specimen, 69 mm. long, contained spermatozoa. Roughly speaking, size up to 68 or 70 mm. indicates sexual immaturity. Animals longer than 70 mm. will generally have the mature organ. Exceptions must be numerous considering the variable length of individuals at metamorphosis (I. Wilder '24).

In a 58 mm. animal identified by Mrs. Wilder as in advanced metamorphosis there are four distinct tubules in the dorsal region of the cloaca. They are in pairs—two on each side of the median line and one pair posterior to the other. These tubules are made up of a very small group of cells closely grouped and darkly stained with barely perceptible lumina and short ducts. The ducts very nearly reach the thick walls of an invagination of the cloacal wall which is the anlage of the central tubule. Several smaller but otherwise similar groups of cells appear more ventral but are most probably not part of the spermatheca group.

A 59 mm. animal in the incipient metamorphic stage also shows two pairs of tubules, one posterior to the other. Although this animal is not as far advanced in metamorphosis as the 58 mm. individual, the spermatheca anlage is in a more advanced condition. The lumina of the tubules are definitely open, the necks curve somewhat dorsally toward the median line and reach the walls of the central tubule. The opening of the central

tubule into the cloaca is minute and the very short duct above is broader in diameter. Columnar epithelium is already present lining this little chamber. There is no sign of the columnar epithelium in the necks or dense cells of the two primary tubules.

A 64 mm. animal, an adult probably recently transformed, shows another pair of tubules and otherwise little change in the spermatheca development, though the cloacal glands are greater in number and length and the cloaca is beginning to form folds of the walls.

In a 66 mm. animal there is another pair of tubules thus making four tubules on a side. The tubules seem to be all of the same size; the blind ends show an open lumen; the necks are solid cords. The central tubule is no larger in size and extent that it was in the 58 and 59 mm. animals but it is further removed from the cloacal wall—more dorsal. There seems to be no opening at all into the cloaca. It is not probable that any sections are lost in this region which might account for not finding an opening. The organ at this time is functionless and is developing slowly; on the other hand the cloaca is developing steadily, that is, the walls are becoming folded and glands are enlarging. The spermatheca group of tubules is left behind until the approach of sexual maturity.

A 68 mm. animal was sectioned horizontally and badly torn by inability to fix the contents of the rectum, but there is discernible the same primitive condition as of the 66 mm. female. The number of tubules has increased; five pairs are certain but six may be present. All are in the same stage. In all these series there is no way of determining the primary pair of tubules nor the latest formed.

From the first few rudimentary tubules with their cord-like necks and small central tubule and the whole without any sign of pigment, to the pigment-walled mature spermatheca which occurs in so many 68–72 mm. females and in practically all of them of greater length, seems a great jump and implies rapid growth in a short period most probably just preceding the first egg-laying season. The author regards it as a piece of good-luck to have found, simply by chance sectioning, as there seems to be no other method of determining age at this period, a series

which is intermediate between the young and the mature spermatheca. This was a 71 mm. female caught and killed in September.

In this spermatheca the average number of tubules found in a mature organ is not complete (though at least one functional organ in this study was observed with no more) and only one pair, one tubule on each side of the median line, is mature in size and cell structure. The necks of these two largest tubules are well developed: the lumina appear at first sight to be open but upon higher magnification they are seen to be so filled with the faintly stained apices of the columnar cells as to be virtually closed tubes. The remainder of the tubules and their necks are in various stages of development.

A most marked development of the central tubule is evident. This seems much out of proportion to the same duct in the fully mature organ. The extent is less, that is, the tube is short and does not extend far posterior, but the diameter is wide. Above the short, stout entrance the duct immediately widens into a very broad chamber into which the necks empty. Most of the necks being rather immature their confluence is not so graceful as is typical in older spermathecæ. Also the cells of the columnar lining of the central tubule have not the length and delicacy of apices peculiar to this duct in all later stages, and consequently they are not so conspicuous.

Lastly pigment is present in quantities but not in the arrangement of the mature organ. Sockets around the ends of the two largest tubules are being formed. Around some of the smaller tubules also it has gathered in tangled networks, but around the largest pair it is more concentrated—coarse, thick strands. Aside from these foci the pigment is scattered and the pigment producing cells still visible. The definitely bounding walls of the organ are not formed.

The spermatheca just described measures slightly less than 1/3 mm. in length and must suffice to complete the developmental series. But a word may be said about the largest spermatheca observed during this investigation. The animal was a 95 mm. female killed in September. Unfortunately for the series as a whole it was sectioned horizontally but the spermatheca is perfect. Eighteen tubules were counted and, although

there is no apparent symmetry in the bilateral arrangement of the tubules, the necks from each side converge separately which causes two reservoirs and these, joining, form the large chamber of the central tubule. This narrows abruptly into the long, narrow dorsal passage which dips into the cloaca.

In this largest spermatheca as in some others which are presumed to be several breeding seasons of age, the central tubule extends quite to the posterior wall of the organ and, coincident, are the exaggerated curves of the necks of the flask tubules. All of the tubules lie in the floor of the organ but the most anterior have their blind end walls faced anteriorly. The necks then curve posteriorly and dorsally. The epithelium in this oldest series is particularly conspicuous and the whole thing is an organ of striking beauty, in size approximately 2/3 mm. long.

III.

Relation to Cloaca.

The development of the cloaca from the simple smooth-walled stage of larvæ to the complications of sexual maturity cannot well come within the scope of this paper, but a brief summary and a short description of the mature conditions should not be out of place in explaining some of the relations of the spermatheca.

The most distinctive feature of the cloaca after metamorphosis, aside from the development of the mucous glands, is the formation of a short, dorsal papilla midway between the vent and the opening of the oviducts. This lengthens antero-posteriorly becoming a fold and also deepens until it appears in sections like an icicle pendent from the roof of the cloaca. Until a period shortly preceding sexual maturity the walls of this fold are comparatively smooth. Then they become folded and especially modified at the tip. The tip broadens into a flat surface with two wings which extend close to the lateral walls of the cloaca so as to form two virtually closed dorsal passages through the cloaca up to the opening of the spermatheca where the entire fold ends. The fold undoubtedly functions as an egg guide causing a kind of continuation of the oviducts into the cloaca, but the function of the two small blind pouches in the posterior region of the fold is unknown.

Although deep folds and spurs in the walls of the cloaca admit of great expansion of the region it does not seem possible that more than one egg could pass at a time, and during the progress of this egg down one side of the cloaca corresponding to the oviduct from which it has proceeded the other passage must be entirely occluded by the pressure of the central fold against the opposite wall. Thus each egg after being squeezed through this crowded region of the cloaca arrives in the larger, freer space at the opening of the spermatheca. The distance from the opening of the oviduct to the mouth of the spermatheca is not over one half a millimeter.

The posterior wall of the fold and an extended area of the dorsal wall of the cloaca surrounding the opening of the spermatheca is lined with the delicate columnar epithelium otherwise singular to the central tubules of the spermatheca. The peculiar function of these cells can be guessed at; they secrete a substance which attracts spermatozoa. During egg-laying the smooth musculature of the spermatheca is probably affected by the convulsions of the entire region and spermatozoa are forced out from the necks of the flasks into the central tubule. Thus disturbed they may swim about and into the cloaca within the secretions of the columnar cells, however, thus preventing any loss. This secretion, as has been stated before, seems not to be mucin or any staining substance but is probably acid. Pfeffer has shown that malic acid is a common attraction for spermatozoa in ferns and Jordan ('91) believes it must be responsible for the attraction of spermatozoa to the spermatheca.

But the substances surrounding the egg as it is released into this region of the cloaca must in some manner offer greater attraction and the egg is probably surrounded with a numerous gathering of spermatozoa. It is not known whether one of these will penetrate the egg membrane immediately before it leaves the cloaca, or whether the whole group will promptly be expelled from the vent, one sperm gaining entrance and the rest perishing during attachment. There is no record of newly laid eggs being examined for impotent spermatozoa adhering to them.

The theory of the attraction of the columnar cells of the area around the mouth of the spermatheca also accounts for the

spermatozoa being drawn up into the spermatheca at the time of reception instead of attempting the larger passages of the cloaca, oviducts or even rectum.

IV.

Summary.

The spermatheca of *Eurycea bislineata* is a compactly walled-in set of tubules in the arrangement of a miniature "cat-o'-nine-tails"; the tails are the bulbed or flask-like storage tubules and the long curved handle is the central tubule which is both entrance and exit for spermatozoa. The organ develops slowly in the median dorsal wall of the cloaca during and after metamorphosis until the autumn before the first egg-laying season, when, more rapidly, the majority of the tubules are formed, pigment appears forming the walls and sockets for the tubules and the columnar lining of the system is completed, proliferating back, probably, from the central tubule.

The author expresses profound appreciation for the help and advice of Dr. Harris Hawthorne Wilder and Mrs. Inez Whipple Wilder in the preparation of this paper.

Material and Technique.

Aside from dissections of fresh and preserved material, fifteen female *Eurycea* were sectioned. In size these vary from 45 mm. to 95 mm. total length. Specimens were killed in September, October, November, February, April and June. All were decalcified and sectioned entire—from the ovary to a point caudal to the vent of the cloaca—to preclude any distortion of the cloacal region. Bouin's fixative was generally used though some of the young animals were preserved in formalin. Fourteen were stained with hematoxylin-eosin and one with muci-carmine. Most of the series are transverse; two are frontal and two sagittal. The drawings are made with a projection apparatus.

ABBREVIATIONS USED.

ant.....anterior	*nc*.....nerve cord
ap.....anterior pouch	*nt*.....notochord
bl.....bladder	*ov*.....oviduct
cg.....cloacal glands	*post*.....posterior
cl.....cloaca	*pu*.....pubis
ct.....central tubule	*pw*.....posterior wall of spermatheca
dor.....dorsal	*rc*.....rectum
f.....dorsal fold of cloaca	*sp*.....spermatheca
fl.....flask tubule	*spz*.....spermatozoa
ms.....mesonephros	*ur*.....ureter
n.....neck	*vent*.....ventral

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EXPLANATION OF FIGURES.

PLATE I.

FIG. 1. (Mag. × c. 85.) Fig. 1 is a diagrammatic drawing of a young functional spermatheca with part of the pigment sac removed. The diagram is based upon the study of the spermatheca of the 69 mm. female of Fig. 3 and the immature spermatheca of a 71 mm. specimen described in the text. An average of eight tubules would be typical of this stage. The necks bend but slightly posterior and some are anterior in direction. At their convergence the central tubule is extremely large but short, antero-posteriorly, and narrows as it dips into the cloaca. The posterior wall and floor of the spermatheca are densely pigmented and sockets enclose the bulb of each flask tubule.

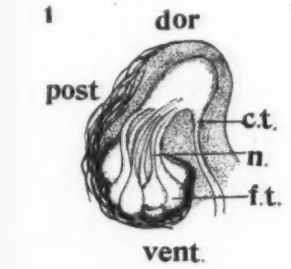
FIG. 2. (Mag. × c. 85.) Fig. 2 is a diagrammatic drawing of an older spermatheca. The sac wall is removed and half of the tubules are shown, bisected to show their contents—the coiled spermatozoa in the flask tubules. These tubules lying in the floor of the sac in pigmented sockets bend posteriorly and dorsally to converge into the central tubule. The reservoir formed by this convergence narrows and the duct passes anteriorly through the dorsal part of the sac where it bends to pass into the cloacal wall. The diagram shows this portion of the central tubule anterior to all the flask tubules but in many cases it passes between the two most anterior flask ends. See Fig. 7. (Mag. × c. 85.)

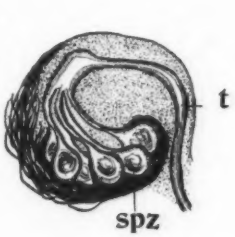
FIG. 3. (Mag. × 19.) Fig. 3 is a para-sagittal section through the region of the rectum and cloaca of a 69 mm. female to show the orientation of the spermatheca. The plane of sectioning is not quite true so that the anterior region of this drawing is very nearly median-sagittal showing the nerve cord, notochord, rectum and bladder. The posterior half of the drawing shows the end of the mesonephros, one oviduct, the spermatheca and the cloaca almost obscured by the many folds of its walls. It is to be noted in this figure that the mesonephros does not extend as far posterior as the spermatheca. This may be compared with transverse sections shown in other figures. In some individuals the mesonephros lies dorsal to the spermatheca and in others it ends more anteriorly. It is not certain whether these variations are due to changes or varying lengths in the mesonephros itself or if they might be due to the influence of the spermatheca.

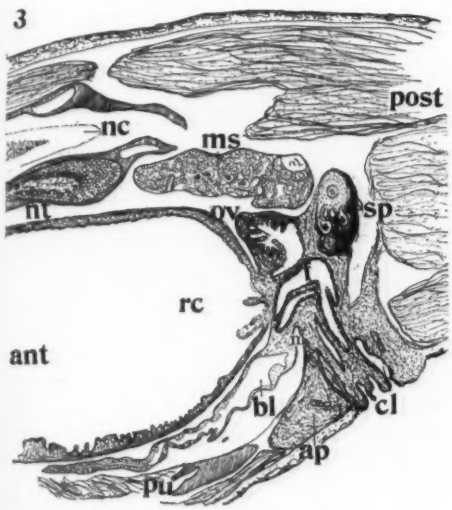
An "*f*" placed below a fold separating the oviduct from the lower part of the cloaca is part of the fold represented in Fig. 10, transverse section.

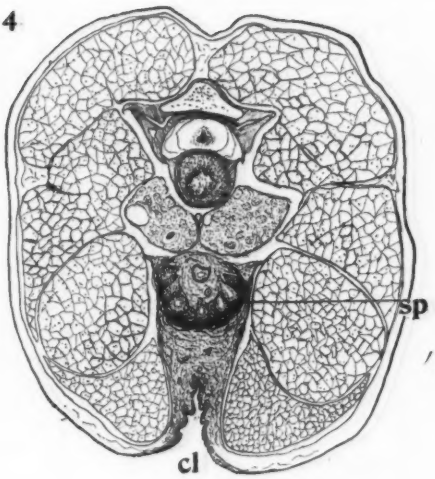
The spermatheca in this specimen is young, of the type represented in Fig. 1. It is higher, dorso-ventrally, than it is long, antero-posteriorly. There are few tubules and the necks of these do not bend posteriorly. Through a median section the central tubule is large as in Fig. 1, but this section is through one side of it. The flask ends are filled with spermatozoa and in this section is the tubule described in the text from which the spermatozoa are streaming into the neck. The lining epithelium of these tubules is flat, unlike the delicate columnar cells pictured in the one flask tubule of Fig. 9, or in Fig. 5.

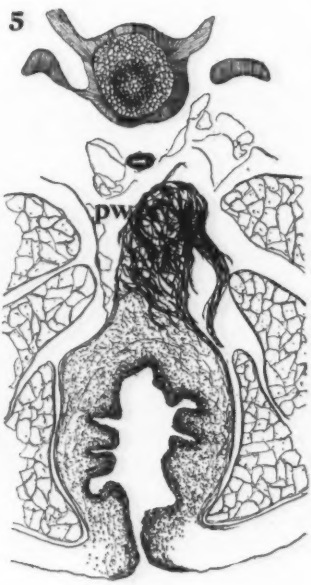
"*ap*" on this figure indicates an interesting fact about the mature cloaca that is very puzzling in transverse sections through the region—an anterior pouch, although this section is only through the wall of it. The anterior pouch is a ventral region of the cloaca separated from the anterior dorsal region by a band of muscle between the bladder and the cloaca. The region is left blank in the figure and indicated by "m." Ventral dissections of the cloaca did not show this particular and in transverse sections it appeared so startling that its function was not guessed.











VERA KOEHRING.

From a sagittal view, however, it is very simply explained. During egg-laying the rectum and bladder are pushed anteriorly, the folds of the cloaca smooth out and the anterior pouch is drawn down as the vent is stretched open. It is but another rather larger fold of the cloaca.

FIG. 4. (Mag. × 17½.) Fig. 4 is a transverse section through a 68 mm. female midway through the spermatheca. The mesonephros lies above the spermatheca. In the floor of the spermatheca lie the flask tubules. A few necks are evident. The central tubule is near the dorsal wall. This is an animal killed in June; the tubules are empty and the columnar cells long. Although this animal is short in body length the spermatheca and cloaca show the maturity of several seasons of breeding.

PLATE II.

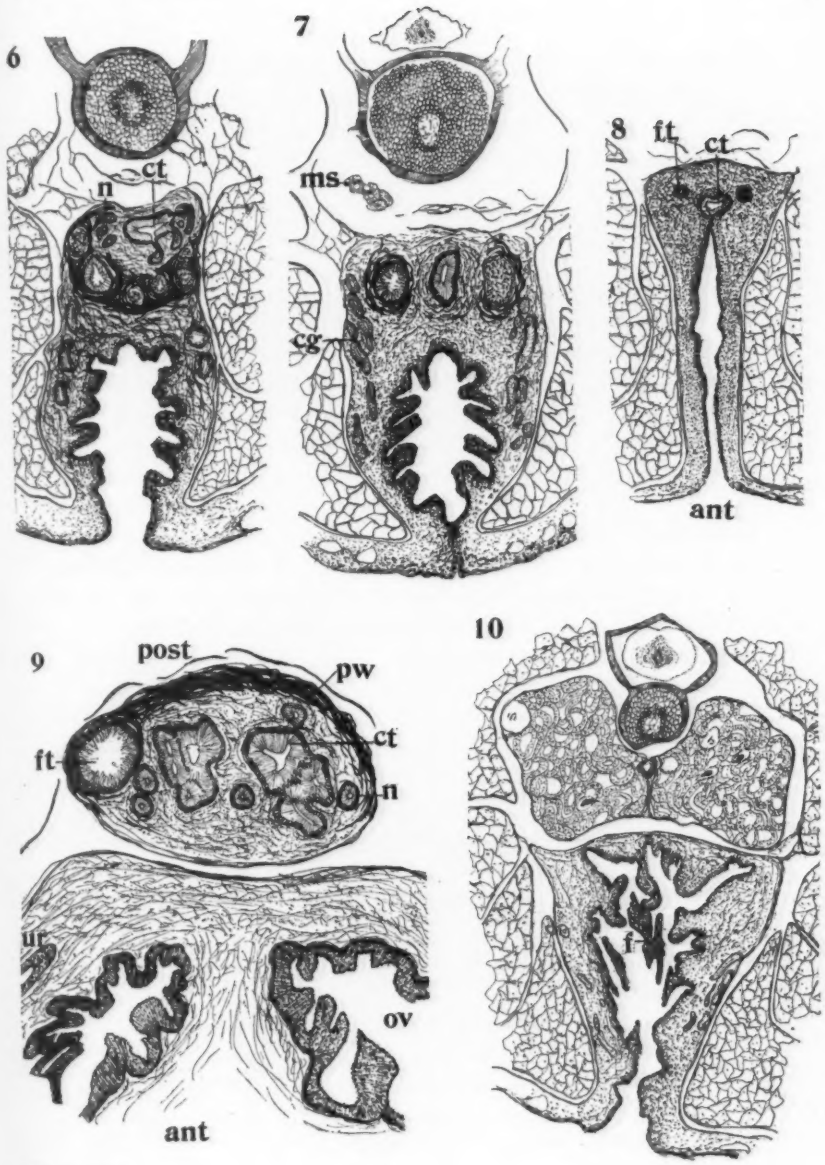
FIG. 5 (mag. × 40), FIG. 6 (mag. × 41½), FIG. 7 (mag. × 42). Figs. 5, 6 and 7 are regions of the spermatheca of a 70 mm. female. This organ has not as many tubules as that of the 68 mm. spermatheca of Fig. 4 nor is the cloaca as complex. On the other hand, there are more tubules and more pigment than in the 69 mm. spermatheca of Fig. 3. The significance of these variations after maturity is not known.

Fig. 5 is a transverse section through the posterior wall of the spermatheca. Fig. 6 is through the region where the necks are converging into the central tubule. Fig. 7 is through the anterior part of the organ; the central tubule bends to open into the cloacal wall and the most anterior flask tubules lie on either side.

FIG. 8. (Mag. × 50.) Fig. 8 is a transverse section through the anlage of the spermatheca in a 59 mm. female. One pair of flask tubules is shown and the central tubule in which the columnar epithelium has developed. The short, cord-like necks do not show in this section.

FIG. 9. (Mag. × 86.) Fig. 9 is a frontal section through a 95 mm. female. The necks converge into two groups which join to form the central tubule. One flask tubule is shown with the characteristic ragged-looking but nevertheless very delicate epithelium. The necks are similar to the epithelium of the central tubule. The oviducts and one ureter are in the anterior part of the drawing.

FIG. 10. (Mag. × 26½.) Fig. 10 is the region through the cloaca of the 68 mm. individual of Fig. 4 several sections anterior to the opening of the spermatheca. The central fold pendent from the wall of the cloaca divides the dorsal region, and serves as an egg guide.



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THE INHERITANCE OF A MACULA MUTATION CONCERNED WITH ELYTRAL SPOTTING AND LATENT TRAITS IN THE MALE OF *BRUCHUS*.[1]

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A conspicuous, bilateral, three-character pattern is found on each elytron of the normal wild type female. It consists of two circular black spots placed one anterior and the other posterior, and a thin horizontal line of white pubescence along the inner edge. The elytron of the wild male is unmarked, the four black spots and the two white lines being absent. This condition enables the sexes to be easily distinguished and makes the wild *Bruchus quadrimaculatus*, Fabr., sex-limited.

In this culture, on October 3, 1922, at the University of Oklahoma, there appeared a male insect bearing indistinct black spots and white pubescence, a pattern similar to that of the female described above although less perceptible. This mutation was designated "macula." Approximately a year of inbreeding and selection was required before homozygous macula cultures could be assuredly separated from the wild type in which they originated, because the macula female and the wild female are identical in both homozygous and heterozygous cultures. The difference between the unmarked wild type male and the spotted, macula male was therefore the only visible criterion available for these selection tests.

The macula mutation is represented homozygously by the genes, MM, and its recessive allelomorph, spotted females and non-spotted males of the normal insect, homozygously by the factors, mm.

In the first test a (MM) female, homozygous for spotted males and females was crossed with a normal (mm) male, homozygous for non-spotted males and spotted females. The offspring from

[1] It is a pleasure to acknowledge my indebtedness to Dr. Frank R. Lillie, Director, for the privilege of utilizing the many excellent facilities of the Marine Biological Laboratory.

22 separate pairs, totaled 422 heterozygous, spotted (Mm) females and 412 heterozygous, spotted (Mm) males. This proves that the macula trait, spotted males and females, is dominant to the normal character, non-spotted males and spotted females. Complete data for this test is tabulated in Table I.

TABLE I.

P_1: 1 MM female, homozygous for spotted males and females × 1 mm male, homozygous for non-spotted males and spotted females.

F_1: Offspring:

Pair No.	Females Spotted.	Males Spotted.	Pair No.	Females Spotted.	Males Spotted.
	(Mm)	(Mm)		(Mm)	(Mm)
3.....	15	16	98.....	15	15
2.....	20	29	99.....	32	42
17.....	28	14	104.....	19	9
19.....	33	19	110.....	1	1
36.....	5	17	116.....	6	5
44.....	38	32	5.....	13	10
51.....	11	13	9.....	0	3
54.....	24	27	10.....	10	0
59.....	24	38	106.....	19	18
60.....	25	16	112.....	27	35
94.....	18	14	120.....	34	39
			Totals.....	422	412

TABLE II.

RECIPROCAL CROSS.

P_1: 1 mm female, homozygous for non-spotted males and spotted females × 1 MM male, homozygous for spotted males and females.

F_1: Offspring:

Pair No.	Females Spotted.	Males Spotted.	Pair No.	Females Spotted.	Males Spotted.
	(Mm)	(Mm)		(Mm)	(Mm)
1.....	11	8	79.....	31	34
10.....	1	2	80.....	37	29
34.....	15	30	81.....	33	16
39.....	11	11	88.....	18	16
47.....	24	19	90.....	24	23
48.....	3	6	92.....	51	56
49.....	15	9	109.....	19	27
63.....	28	46	123.....	22	25
64.....	6	7	6.....	2	0
68.....	7	6	9.....	9	5
69.....	15	16	7.....	49	27
78.....	44	46	8.....	12	9
			Totals....	488	483

TABLE III.

F_1: Mm heterozygous spotted female × Mm heterozygous spotted male.
F_2: Progeny:

Pair No.	Females Spotted.	Males.	
		Spotted.	Non-spotted.
	(MM, 2 Mm, mm)	(MM, 2 Mm)	(mm)
4	23	19	9
87	37	30	7
126	15	10	3
127	45	33	15
128	30	19	4
11	14	16	4
3.1	26	15	6
3.4	7	6	1
3.7	48	31	13
19.1	9	11	3
19.9	34	20	5
44.3	36	27	8
54.15	22	16	3
54.31	32	25	7
71.5	4	5	2
87.3	63	44	15
99.5	16	13	3
104.3	17	18	5
116.1	8	4	1
116.3	17	12	3
2.6	21	17	8
2.7	9	7	2
54.20	33	29	9
59.6	54	37	14
60.6	13	11	4
5	49	39	16
20	37	22	9
62	7	5	2
63.1	75	58	20
69.1	21	17	2
69.5	20	15	5
81.5	26	12	3
91.2	43	39	19
108.4	55	45	16
109.3	27	28	8
109.4	69	59	22
123.2	34	35	10
123.3	31	20	5
123.7	79	68	16
7.2	21	17	4
7.3	24	20	6
7.4	7	8	2
6.2	19	12	4
Totals	1,079	994	323

The second experiment was the reciprocal of the previous one. A (mm) female, homozygous for spotted females and non-spotted

TABLE IV.

BACK CROSS TEST

P_1: Mm heterozygous spotted female × mm homozygous non-spotted male.
F_1: Progeny:

Pair No.	Females Spotted.	Males.	
		Spotted.	Non-spotted.
	(Mm, mm)	(Mm)	(mm)
4	23	13	10
71	31	19	20
86	32	16	17
96	10	4	3
105	0	1	2
119	22	7	7
125	21	17	16
3.1	26	13	10
3.2	32	9	10
3.5	10	7	6
3.6	30	16	10
17.3	12	7	4
198	18	11	15
51.4	3	2	3
51.6	36	18	13
54.1	20	22	23
54.2	45	20	12
54.4	25	14	13
54.14	7	4	3
54.16	37	18	18
54.30	28	10	9
60.1	131	47	69
60.3	31	11	19
60.4	27	14	14
60.8	43	22	23
60.9	30	13	19
85.3	34	20	16
86.2	74	40	52
87.1	126	68	50
94.4	24	7	11
94.5	13	4	6
98.2	31	5	6
98.3	29	10	12
99.1	23	17	10
99.2	103	34	34
99.3	4	3	3
99.4	96	44	42
119.1	11	7	9
125.3	37	18	11
127.1	52	24	24
128.4	11	3	4
Totals	1,375	679	658

males was bred to a (MM) male, homozygous for spotted males and females. The F_1 offspring from 24 separate pairs gave 488

heterozygous, spotted (Mm) females and 483 heterozygous, spotted (Mm) males. This test further shows the dominance of the spotted trait to the normal. Furthermore this experiment, when compared with the previous one, proves that the character is not sex-linked. Table II. gives the data.

The third test concerned heterozygous, spotted (Mm) females and heterozygous, spotted (Mm) males. The F_2 progeny from 43 F_1 single pairs totaled 1,079 spotted (MM, Mm, mM, mm) females, 994 spotted (MM, Mm, mM) males, and 323 non-spotted (mm) males. The result approaches a 4 : 3 : 1 sex-limited ratio, which shows that the spotted females appear identical, and that macula is dominant to normal. This data is tabulated in Table III.

The fourth experiment is a back cross with the F_1 hybrids, (Mm), heterozygous insects, and the normal type (mm) weevils. The parents were heterozygous, spotted (Mm) females and homozygous, non-spotted (mm) males. From 41 single pair matings 1,375 spotted (Mm, mm) females, 697 heterozygous, spotted (Mm) males, and 658 homozygous, non-spotted (mm) males, were obtained. The ratio, therefore, approximates a sex-limited 2 : 1 : 1. This result indicates further that normal spotting is recessive to macula. The data is listed in Table IV.

The fifth experiment was another back-cross test, the reciprocal of the previous one. Homozygous, normal, spotted (mm) females were mated with heterozygous spotted (Mm) males. From the 43 different pairs a total of 1,415 spotted (Mm, mm) females, 786 heterozygous spotted (Mm) males and 798 homozygous non-spotted (mm) males were obtained. This gave a 2 : 1 : 1 sex-limited ratio, or actually a 1 : 1 ratio, since the females appeared alike. Spotting is dominant to the normal type. Complete information is found in Table V.

In the sixth test heterozygous spotted (Mm) females and homozygous spotted (MM) males were used. The offspring from 26 separate pairs totaled 701 spotted (MM, Mm) females and 654 spotted (MM, Mm) males. This demonstrates the dominance of the macula character to its recessive normal trait, spotted females and non-spotted males. This data is compiled in Table 6.

TABLE V.

BACK CROSS TEST: RECIPROCAL CROSS.

P_1: mm homozygous wild type female × Mm heterozygous spotted male.
F_1: Offspring:

Pair No.	Females Spotted.	Males.	
		Spotted.	Non-spotted.
	(Mm, mm)	(Mm)	(mm)
6	33	30	27
6.1	2	4	4
31	27	20	15
91	33	18	19
108	20	8	9
100	24	10	13
103	41	24	19
1.2	34	14	15
20.7	16	8	9
31.6	31	19	26
34.3	16	9	6
34.2	11	3	4
39.1	25	16	19
78.1	31	33	38
78.3	17	4	4
79.1	29	11	17
79.3	48	19	19
79.7	13	6	4
81.4	33	15	18
88.2	62	33	33
88.7	15	7	11
91.1	31	16	14
91.3	95	37	34
91.6	34	60	51
92.1	15	7	12
92.5	73	22	28
92.6	157	83	78
108.1	5	7	12
123.1	67	28	29
123.4	40	19	16
64.1	6	7	5
64.2	8	10	11
112.1	33	13	16
112.2	64	37	31
9.10	42	19	23
54.11	3	2	3
54.28	30	13	17
82.2	25	15	11
116.5	29	12	10
128.1	27	23	17
10.10	8	4	5
101.2	15	10	10
Totals	1,415	786	798

The seventh experiment was the reciprocal of the previous one. The parents were homozygous, spotted (MM) females and

TABLE VI.

BACK CROSS TEST.

P_1: Heterozygous, Mm, spotted female × homozygous, MM, spotted male.
F_1: Offspring:

Pair No.	Female Spotted.	Male Spotted.	Pair No.	Female Spotted.	Male Spotted.
	(MM, Mm)	(MM, Mm)		(MM, Mm)	(MM, Mm)
20.3.....	1	1	47.5.....	19	17
48.2.....	4	5	49.1.....	21	24
62.4.....	17	15	49.2.....	3	4
69.4.....	9	4	47.7.....	5	9
78.6.....	70	56	47.8.....	30	34
80.5.....	3	3	48.1.....	11	6
81.1.....	33	16	68.5.....	31	32
88.5.....	3	1	68.6.....	28	40
88.6.....	22	5	68.7.....	29	25
92.7.....	131	149	79.5.....	37	32
47.9.....	37	25	80.2.....	21	30
47.3.....	43	34	80.4.....	36	49
47.4.....	28	20	81.8.....	29	18
			Totals....	701	654

heterozygous spotted (Mm) males. The offspring from 15 pairs, gave 236 spotted (MM, Mm) females and 213 spotted (MM, Mm) males. Hence, both sexes were homozygous for the macula (MM) genes, and heterozygous for the macula (M) gene and normal (m) factor. The macula mutation is dominant to the wild type. Complete data is presented in Table VII.

TABLE VII.

BACK CROSS TEST.

Reciprocal Cross.

P_1: Homozygous, MM, spotted female × heterozygous, Mm, spotted male.
F_1: Offspring:

Pair No.	Female Spotted.	Male Spotted.	Pair No.	Female Spotted.	Male Spotted.
	(MM, Mm)	(MM, Mm)		(MM, Mm)	(MM, Mm)
17.1	28	23	94.6	10	5
17.2	4	1	51.8	6	9
51.2	21	10	54.12.....	3	0
51.3	2	2	54.21.....	36	33
51.11.....	18	18	126.4	9	17
59.2	26	18	126.8	18	18
59.3	23	27	101.	29	31
71.4	3	1			
			Totals.....	236	213

The above series of experiments prove that the two elytra traits, normal and macula, differ in the male phenotypes only, since the factors for the macula (MM) mutation and the wild (mm) type appear identical for both female traits. These females are alike phenotypically but deviate genotypically, since the normal female has the same black four spotted pattern as its mutant, the macula female. Again, normal is recessive to macula.

Many sex-differences are detected in insects, occurring most frequently in Diptera; *Drosophila* probably furnishing the greatest number, with Lepidotera next, then Coleoptera. This sequence appears, as the result of the amount of genetic study directed upon these species. Many of the sex-limited traits in *Drosphila* are less distinct in one sex than in the other, thus differing somewhat from *Bruchus*, in which there is no visible manifestation of such characters in the male, except for the macula mutation.

Examples of these sex-limited traits for *Bruchus*, in which the male elytra is a non-spotted tan, have been previously demonstrated, hence they will be merely mentioned in this relation. The first mutations discovered (Breitenbecher, 1921) consisted of red, black, white, and tan elytra colors apparent only in the females. The respective male for each of these four female cultures was a non-spotted tan, the wild type elytra color. The four traits are multiple allelomorphs. A second non-visible trait in the male occurs with mosaic females (Breitenbecher, 1922). These females have elytra of different colors, often combined with varied spotting. In a third instance the males remained non-spotted tan, although the females displayed four red spots on the elytra (Breitenbecher, 1923). This character was dominant to the normal. Another mutation, in which the male showed a complete absence of the trait, was that in which all females were apterous. The males were fully winged. This was a recessive character (Breitenbecher, 1925). Lastly, another mutation, never visible in the male, was called "piebald." Here bilateral asymmetry was manifested since the females were of two types, about equal in numbers. One group had two red spots on the left elytron and two black spots on the right; the

other group had two black spots on the left elytron and two red ones on the right. When the two types of females were added, the trait was found to be inherited as a recessive to normal (Breitenbecher, 1925). Every male, for the entire list of characters enumerated, had non-spotted tan elytra similar to the wild type.

It is improbable that such latent characters in the male are caused by developmental differences, because in sex-limited, as well as other Bruchid mutations, the male emerges a fraction of a day before the female. Inhibitors associated with the male complex may produce this peculiarity. Or, since the female has two X-chromosomes, and the male only one X-chromosome, the sex-limited traits may be caused by pattern, or normal, genes within the X-chromosome. These may be associated with factors in other chromosomes in a degree enabling the character to be completely manifested in the female. The one sex-chromosome of the male may not be sufficient for complete manifestation of the trait. This conception is similar to that of Bridges (1922) for *Drosophila*.

The macula and normal patterns of *Bruchus*, illustrating sex differences, may be considered as the result of identical phenotypes for the female, since the females for each trait have duplicate spotting patterns on the elytra. The mm genes in the wild male are non-visible, while the MM factors for the normal four black spots are visible in the macula male.

It is to be hoped that some mutation may occur, in which the gene concerned with the entire pattern or non-pattern trait may appear. This might assure a definite solution for these sex-limited differences in *Bruchus*.

Conclusions.

1. The macula mutation is dominant to the wild type.
2. Genes, mm, represent the wild type, having spotted females and non-spotted males.
3. Factors, MM, represent the macula mutation, in which both males and females are spotted.
4. The macula character is not sex-linked.
5. Inhibitors probably produce latent traits in the male.

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THE EFFECT OF THYROID FEEDING ON THE MOULTING PROCESS AND FEATHER STRUCTURE OF THE DOMESTIC FOWL.

HARRY BEAL TORREY AND BENJAMIN HORNING.

1.

In the course of investigations on some of the endocrines and their functional correlations in the domestic fowl, it was observed that the addition of desiccated thyroid to the ration of Brown Leghorn and White Leghorn chicks led to striking modifications in the form of the first set of rectrices, and in the course of the first moult (Horning and Torrey, 1923). The form of the rectrices was modified conspicuously by the shortening of the vane and the lengthening of the naked region of the shaft. A characteristically "quilled" feather was the result, though somewhat reduced in total length. The course of the moult was modified by the maintenance of base-to-tip continuity between first and second rectrices, in sharp contrast to the normal independent and discontinuous development of these feathers.

The results of this experimentally produced hyperthyroidism, and others to be considered in another place, were then examined for the light they might throw upon the rôle of the thyroid hormone in feather differentiation, possibly in processes of differentiation in general. Feathers afford certain advantages as material, for they give permanent records of the progressive changes that occur in the feather germ during their development. But it is only of such a period that they do give reliable records, obviously enough, for once grown, they are removed from the influence of experimental procedures to which they might earlier have been susceptible.

2.

The following experiment is typical and with the accompanying tabulation will suffice to establish the fact that desiccated thyroid given in appropriate doses produces "quilling" of the rectrices of chicks of both sexes, with or without gonads.

Fifty-two Brown Leghorn chicks, hatched March 27, were divided into four groups as indicated in Table I.: two control groups (one with and one without gonads); two experimental groups (one with and one without gonads). A week later, on April 3, the rectrices of all were pushing out. On that date daily doses of 16 mgm. of desiccated thyroid (Armour's, containing 0.2 per cent. iodine) in capsule by mouth, were begun for one group of normal chicks (Nos. 36–50). On April 17, the dosage was increased to 32 mgm. and given to the second experimental group (Nos. 51–63) also.

TABLE I.

Controls.			Thyroid-fed Chicks.		
No. of Chick.	Sex.	Rectrices.	No. of Chick.	Sex.	Rectrices.
25	Female	None quilled	37	Male	2 pairs quilled
26	Female	" "	38	Male	4 " "
27	Male	" "	39	Female	6 " "
28	Female	" "	40	Female	4 " "
29	Female	" "	41	Female	2 " "
30	Male	" "	42	Female	5 " "
31	Male	" "	44	?	4 " "
32	Male	" "	45	Male	4 " "
33	Male	" "	46	Female	4 " "
34	Female	" "	47	Male	3 " "
35	Male	" "	48	Male	3 " "
36	Female	" "	49	Male	2 " "
			50	Fmeale	5 " "

Gonadless Controls.			Gonadless Thyroid-fed Chicks.		
No. of Chick.	Sex.	Rectrices.	No. of Chick.	Sex.	Rectrices.
64	Male	None quilled	51	Male	3 pairs quilled
65	Female	" "	52	Male	4 " "
66	Female	" "	53	Female	? ? ?
68	Female	" "	54	Male	6 pairs quilled
69	Male	" "	55	Male	1 " "
70	Male	" "	56	Female	2 " "
71	Male	" "	57	Male	? ? ?
72	Female	" "	58	Female	? ? ?
73	Female	" "	59	Male	? ? ?
74	Female	" "	60	Male	6 pairs quilled
75	Male	" "	61	Female	1 " "
76	Female	? ?	62	Female	3 " "
77	Male	None quilled	63	Female	1 " "
78	Female	" "			

The condition of the rectrices in all groups on May 18, 52 days after hatching, is shown in Table I.

The shortening of the vane characteristic of the "quilled" feathers was due to a failure of a considerable number of barbs to differentiate proximally. There was no sign of a loss of barbs previously formed and broken off. The vane of each "quilled" feather, though abnormally proportioned as already indicated, ended below in an aftershaft of barbules surrounding the superior umbilicus, with no sign that there had ever been barbs proximal to this point.

As is well known, the barbs of a feather arise from the so-called intermediate cells of the feather germ epidermis, these intermediate cells differentiating in the growing zone into parallel ridges, which, as the feather germ elongates, run a longitudinally oblique course about the dermal core. These ridges differentiate beginning with their distal ends into the barbs and barbules of the definitive feather and are continually renewed from below.

Now the effect of thyroid feeding was to abbreviate the period of ridge formation in the growing zone, and as a consequence to suppress prematurely in that region the processes of differentiation on which barb formation normally depends, without suppressing, however, the processes on which the lengthening of the calamus depends. It is true that the total length of each of the "quilled" feathers thus produced was somewhat less than normal, but that this was not to be attributed to a direct inhibitory effect of the thyroid upon the growth of the feather germ, but rather to a precocious development of the second rectrices, will appear, we believe, from facts to be given below. It may be said here, however, that while the suppression of barb ridge differentiation occurred prematurely after thyroid, in rare cases these ridges might appear again prematurely, after thyroid, about the inferior umbilicus of the first rectrix, foreshadowing the base-to-tip connection of first and second rectrices, to be described in the next section.

3.

In size, shape and coloration—details of which do not here concern us—the first rectrices, belonging to the first few weeks of the chick's life after hatching, differ distinctly from the second, which are adult in type.

FIG. 1. White Leghorn male about 8 weeks old, with normal rectrices On the extreme right is a rectrix of the first set; all the others are of the second set.

Normally the first or infant rectrices may drop away without urging from below; or they may be pushed out by the seconds that are developing from the same follicles. In neither case, however, are the two continuous. Though the succeeding

feather may arise from the same germinal papilla as its predecessor, a period of inactivity of the papilla follows the withdrawal from the calamus of the first and the rounding off of the inferior umbilicus. A new feather is then begun, within a sheath of its own.

The rounding off of the tip of the calamus of the first feather and the establishment of a discontinuity between the first feather and its successor involve certain processes of differentiation before the feather follicle and papilla become quiescent. One of the effects of thyroid feeding in our experiments was to suppress these terminal processes of differentiation by maintaining the papilla in continuous, even though, it may be, reduced activity. This continuity of process was revealed in continuity of structure —and, incidentally, settled, in this case, the question that has sometimes been raised whether successive feathers from the same follicle spring from the same papilla. Here the second obviously did spring from the same papilla as the first. If the feathers of succeeding moults arise similarly, the series, in place of a single feather, would appear to be due to a rhythmic variation in the velocity of critical reactions conditioning the development of the papilla. This variation, normally extreme and leading to discontinuity, is essentially independent of the normal thyroid secretion. The addition of thyroid to the ration under the conditions of our experiments lessened the amplitude of the rhythmic variation without determining the rhythm itself.

The "quilled" first rectrices were originally noticed in our thyroid-fed chicks as distal appendages to the members of the second set, since they were not being shed in normal fashion. Examination showed that the structure of the inferior umbilicus was unusual, the proximal end of the calamus being wide open and the plane of the orifice running ventrodorsally at a sharp angle with the axis of the calamus. Dorsally the lip of this oblique orifice was extended proximally as though the quill had been cut to form a long pen point.[1]

[1] In exceptional cases, as in a wing quill before us, this proximal extension is not a prolongation of the median dorsal line of the calamus, but of a region perhaps twenty degrees away from it. Though the median dorsal lines of the two feathers do not lie in the same plane, there is no obvious structural twist to account for the fact.

At the tip this penlike extension was continuous with the rhachis of the proximal feather. Elsewhere the lips of the orifice, including the edges of this dorsal extension, were continuous with the distal ends of about fifteen pairs of barbs of the proximal feather. The original orientation of the barbs, which had differentiated from long spiral ridges in the wall of the cylindrical feather germ, was thus preserved, and the orifice correspondingly obscured.

The falling of the distal feather was accomplished by rupture of these connections. As a result, the distal end of the second rectrix presented a frayed appearance. This came to be recognized as a characteristic of the second rectrices of thyroid-fed birds, in sharp contrast with the smooth rounded ends of the vanes of normal birds.

4.

What had been said regarding the effect of thyroid feeding on the development of the first rectrices and the first moult applies also to wing quills, though much less frequently and with slight unessential differences in detail; such, for instance, as the obliquity of the umbilical orifice. Contour feathers are not affected, according to our observations.

The facts may be briefly summarized. Thyroid feeding inhibits: (1) the differentiation of proximal barbs and barbules, thus shortening the vane without proportionately shortening the calamus; the proximal region of the vane passing into the after shaft normally without traumatic defect; (2) the usual differentiation of the inferior umbilicus; (3) the withdrawal of the feather pulp from the calamus, and the normal inactive period of the pulp. Continuity between first and second rectrices is the result.

Thyroid feeding also (4) accelerates the differentiation and eruption of the second rectrices. This acceleration was observed first in Rhode Island Red males (Torrey and Horning, 1922). That it occurs in Brown Leghorns is shown by the facts in the next paragraph. The precocious development of the second rectrices correspondingly limits the maximum growth period of the first and probably accounts, at least in part, for the reduced length of the latter after thyroid.

Thirty-two Brown Leghorn chicks hatched April 9 were divided into two lots, each containing 8 males and 8 females. One lot was fed desiccated thyroid, beginning April 14: 10 mgm. daily for 5 days, then 15 mgm. daily. On May 22, 43 days after hatching, the moult had proceeded as shown by the following table.

TABLE II.

Controls.				Thyroid-fed Chicks.			
No. of Chick.	Sex.	First Rect.	Second Rect.	No. of Chick.	Sex.	First Rect.	Second Rect.
1....	Female	4 pairs	3 pairs	17.....	Female	1 + 3 pairs	4 pairs
2....	Female	5 "	1 "	18.....	Female	3 + 4 "	4 "
3....	Female	7 "		19.....	Female	3 "	3 "
4....	Female	7 "		20.....	Female	1 + 2 "	4 "
5....	Female	5 "	2 "	21.....	Female	1 + 4 "	4 "
6....	Female	7 "		22.....	Female	3 "	4 "
7....	Female	7 "	1 "	23.....	Female	1 + 3 "	4 "
8....	Female	5 "	2 "	24.....	Female	4 "	3 "
9....	Male	7 "		25.....	Male	6 "	1 "
10....	Male	7 "		26.....	Male	3 + 4 "	3 "
11....	Male	7 "	1 "	27.....	Male	5 "	2 "
12....	Male	6 "		28.....	Male	5 "	2 "
13....	Male	6 "		29.....	Male	2 "	4 "
14....	Male	0 "		30.....	Male	4 "	3 "
15....	Male	7 "		31.....	Male	died	
16....	Male	7 "		32.....	Male	3 pairs	4 "

The second rectrices recorded for the controls were just appearing. Those recorded for the thyroid-fed chicks were an inch or more longer. In chicks numbered 17, 20, 21 and 23 the variation in the notation indicates that one pair of first rectrices was "quilled" and continuous with the second; in number 26, 3 pairs were "quilled" and similarly continuous with the second.

5.

In certain respects the modifications in structure and moulting that we have described recall defects of structure and moult that have been associated with fault-barring through the work of Strong (1902), Riddle (1907, 1908, 1908*a*) and Whitman (1919).

Strong (1902) called attention to abnormal transverse bands in both remiges and rectrices of a hybrid pigeon, referable clearly to defective barbules. He also described and figured one of the body coverts, for the most part downy and carrying a

FIG. 2. White Leghorn male about 7 weeks old, that was fed thyroid from the age of two weeks. The outer pair of infant rectrices are not "quilled" and may be compared with their "quilled" mates intervening.

tassel-like distal appendage with a horny cylindrical base continuous with rhachis and barbs of the main body of the feather. The horn cylinder he interpreted as a region in which, during

the development of the feather, differentiation but not cornification had been omitted.

Riddle (1907) distinguished five types of defect in feather structure, two being represented by Strong's cases, a third by a very inconspicuous transverse depression or line without defects in barbules, a fourth by constrictions in the feather germ, and a fifth by a single instance of a feather with barbs broken off at even distances from the shaft, the defect extending parallel to the rhachis.

The first three of these are of widespread occurrence among birds. Riddle discussed them in connection with what Whitman had called fundamental bars (alternating lighter and darker transverse bands of pigment) so beautifully figured in his posthumous paper (1919, pls. 72–75). He concluded (1908) on the basis of experiments, that both are referable to variations in the nutrition of the feather germ during its development, the fundamental bars being produced by diurnal changes in blood supply, the fault bars by defective nutrition through this and other agencies. In his experiments, reducing the food, feeding Sudan III, mechanical destruction of tissues and blood vessels and the administration of amyl nitrite were all effective.

We have no satisfactory evidence of variation in the blood pressure of our birds. But we have seen abnormalities in feather structure belonging to Riddle's fifth type as a direct consequence of starving two White Leghorn males. One of our adult male Brown Leghorns on a daily ration of 1 gm. of dessicated thyroid replaced three pairs of remiges with feathers of normal length but abnormal in structure and color. The vanes were narrower and proximally misshapen as though fashioned under cramped conditions. The normal color was almost entirely lacking, the feathers being prevailingly white. There appears to be no reason to doubt that directly or indirectly such defects are referable to disturbed nutrition. We have also two rectrices from an adult Rhode Island Red female nearly two years old that resembles Strong's feather with a "tassel-like appendage." One of these rectrices is marked with great regularity by transverse depressions belonging to Riddle's third type of feather defect. At the distal end of the "appendage" two or three of these are definitive

fault bars, being represented by actual defects in barbule formation. Toward the base of this "appendage," as the vane narrows, they become more and more closely spaced. When the vane broadens out again, beyond this narrow neck, these lines are less closely but very regularly spaced. Assuming that the distance between successive lines is a measure of the daily growth of the feather, the setting off of distal appendage or lobe from the rest of the feather was correlated with a period of diminished growth velocity due to a cause not definitely known in this case, but very possibly a nutritional deficiency covering about fifteen days.

Riddle (1908*a*) interpreted in a similar way the relation between down and succeeding definitive feathers. Jones (1907) had shown that the first down and its succeeding definitive feather are produced by one continuous growth, the former being the plumulaceous tip of the latter. But the down feather is differentiated from the pennaceous feather, with which it is continuous, by a short quill, which Riddle (1908) regarded as a defective region due to the same general cause responsible for fault bars, namely, inadequacy of nutrition. True quills also, Riddle thought, resulted from a diminished food supply.

There are, then, certain points of resemblances between these phenomena which, whether normal or abnormal, have been interpreted as results of variations in the food supply, and the phenomena we have described as results of thyroid feeding. There are, however, certain significant points of difference.

Thyroid feeding shortens the vane of the first rectrix in chicks and increases the proportionate length of the calamus by the suppression of proximal barbs and their associated barbules. This resembles effects attributed to poor nutrition.

Thyroid feeding tends to maintain continuity between first and second generations of rectrices. We have no evidence that poor nutrition does this.

Thyroid feeding tends to accelerate the differentiation and eruption of the second rectrices. Poor nutrition, on the contrary, tends to retard these processes.

It appears then that these results of thyroid feeding are of two opposing sorts, only one of which is comparable with the

FIG. 3. Three wing feather from young Brown Leghorns showing continuity of feathers in succeeding generations as a result of thyroid feeding. Only a portion of each second generation feather is shown (below).

effects of nutritive deficiency. Such effects vary in intensity with the dose of thyroid administered. The "quilling" in the

birds recorded in Table I., more pronounced than in the birds of Table II., followed a heavier dosage of thyroid. And after the exceptionally large daily ration of 1 gram of dessicated thyroid one of our adult males already mentioned replaced 3 pairs of moulted remiges with feathers the proximal portions of whose vanes presented the deformities that characteristically follow starvation. There were pigmentation defects also, but these will be considered at another time.

The second consequence of thyroid feeding, namely, the acceleration of feather differentiation and eruption, would seem to involve an acceleration of division processes in the cells of the feather germ. According to Champy (1922) thyroid added for twenty-four hours to water containing frog tadpoles accelerates cell division in certain zones and tissues far beyond normal velocities. Ebeling (1924), using much more satisfactory methods, has established the accelerating effect on cell division of substances secreted by living thyroid which is added to cultures of fibroblasts *in vitro*. Whether thyroxin would act similarly is not yet known. Thyroxin (Squibb's) does not accelerate the division rate of *Paramecium*, but, on the contrary, in dilutions of 1 : 1,000,000 actually depresses it (Riddle and Torrey, 1923; Torrey, 1923, 1924; Torrey, Riddle and Brodie, 1925). The evidence from our present material will be offered at a later date.

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ON THE ACTION OF CERTAIN SUBSTANCES ON OXYGEN CONSUMPTION.

VI. The Action of Acids.

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In recent years the rôle of acidity and alkalinity in biological processes has been the subject of numerous investigations. The impetus to this field of research was given by the invention of methods for determining the true hydrogen ion concentration of biological fluids and materials. As a result of the great mass of work accumulating along this line of research, there prevails among biologists the impression or opinion that hydrogen ion concentration is of tremendous importance in the life of organisms. Yet, looking back upon the history of science, one may be pardoned for a certain degree of skepticism. Biologists appear prone to attach undue significance to whatever field of investigation happens to be the fashion of the decade and time alone can assign a mass of research on one particular topic to its proper place.

The present series of experiments was undertaken in part as a test of the proposition that hydrogen ion concentration is of fundamental importance in physiological processes. The consumption of oxygen in respiration was chosen as a physiological process essential to the organism in the highest degree and readily susceptible to quantitative measurement. An attempt was made to determine: (*a*) whether increasing the acidity of the environment of an organism has any effect upon the rate of oxygen consumption; (*b*) whether this effect is assignable to the free hydrogen ions or to some other factor. The experiments prove that alterations in external acidity markedly affect the rate of respiratory metabolism of animals; but they also show that the free hydrogen ions are little, if at all, responsible for the observed effect.

Literature.

Surprisingly enough, very few investigations have been carried out on the effect of increased acidity on the respiratory metabolism of organisms. A rather careful search through the iterature has revealed only the following researches.

Among plants, only the lower forms have been investigated. Brooks ('21, '22) measured the carbon dioxide production of *Bacillus butyricus*, *B. subtilis*, and *B. tuberculosis* at various hydrogen ion concentrations. The bacteria were tested in dextrose solutions made in distilled water and the desired acidity was obtained with sulphuric acid. The rate of carbon dioxide production was at the maximum at pH 7.0 for *B. butyricus*, at pH 6.8 for *B. subtilis*, and was decreased at all acidities greater than these. In the case of *B. tuberculosis*, however, the carbon dioxide production was constant at all acidities between pH 7.4 and 4.4 and was decreased only at acidities greater than 4.4. Gustafson ('20) studied the effect of altered acidity on the respiration of the mold *Penicillium chrysogenum*. Variations in acidity between pH 8.0 and pH 4.0 were without effect upon the rate of carbon dioxide production. In acidities ranging from pH 4.0 to 2.65, the carbon dioxide output first rose and then fell; below 1.95 an irreversible decrease in the output was observed. The oxygen consumption was measured at pH 2.0 and was found to be markedly increased over the normal, thus agreeing with the initial carbon dioxide production at this particular acidity. In Gustafson's experiments, the mold was tested in dextrose solutions in distilled water and sulphuric and phosphoric acids were employed to increase the acidity.

Among animals, a few researches are available on eggs, tissues, and intact animals. Loeb and Wasteneys ('11) found that the oxygen consumption of sea-urchin eggs is slightly decreased in sea-water acidified to pH 6.0 to 4.0, and considerably decreased at or below pH 4.0. Thunberg ('09, '10) has carried out a large number of experiments on the action of acids on the rate of oxygen consumption and carbon dioxide production of surviving frog's muscle. The tests were performed in salt solution. Thunberg unfortunately did not determine the pH of his solutions—this procedure was not customary at that time—but gives the

concentration of acid in terms of molar strength. Hydrochloric acid decreased both oxygen consumption and carbon dioxide output of the muscle markedly, the decrease varying from 15 per cent. in 1/200 mol. HCl to 72 per cent. in 1/20 mol. HCl. Thunberg also tested the action on gaseous exchange of the frog's muscle of a large number of organic acids, including mono-, di-, tri-, and oxycarboxylic acids of both the paraffin and olefin series. Each acid was tested in the following concentrations: 1/100, 1/25, 1/10, and 1/5 molecular strengths. In the majority of the acids, both the oxygen consumption and carbon dioxide production were decreased at all concentrations but not very greatly so. The maximum decrease with the strongest concentrations was 15–25 per cent. In some of the acids (*e.g.*, propionic, butyric, lactic), the respiratory rate was increased in the more dilute concentrations, decreased in the stronger solutions. In fumaric, malic, and citric acids, the carbon dioxide output was greatly increased, while the oxygen consumption was but little altered. On the other hand, oxalic and malonic acids were found to decrease the carbon dioxide output to a much greater extent than the oxygen consumption. Succinic acid alone decreased the former while increasing the latter. It must be remarked that the concentrations used by Thunberg are extremely high and it is difficult for me to believe that the muscle could have remained uninjured in any of the solutions employed by Thunberg, even though each experiment lasted for only thirty minutes. Exposure to 1/50 or 1/20 mol. HCl, for instance, concentrations used by Thunberg, is, I believe, rapidly fatal to any living tissue. Thunberg does not consider the possibility of injury to the muscle in his experiments and makes no statements concerning the condition of the muscle during or after the exposure. I do not believe his experiments can be accepted as of physiological significance. Gray ('24) has studied the action of acids on the oxygen consumption and activity of ciliated tissues of bivalves. Acetic, butyric, and hydrochloric acids were used, presumably in sea-water. In the acidified water, both ciliary activity and oxygen consumption were diminished and in relation to the amount of acidity. Acetic acid at a pH of 4.6 to 4.2 and butyric acid at 4.2 caused a very great decrease and even complete

abolition of oxygen consumption. Hydrochloric acid was less effective than either of the two organic acids, producing only 50 per cent. depression at pH 3.0.

There is a dearth of experiments on entire animals. Jewell ('20) tested the carbon dioxide output of frog tadpoles in distilled water acidified with HCl. The output was decreased to 92 per cent. of the normal at pH 7.0, to 84 per cent. at pH 5.4, to 53 per cent. at 3.8, and to 37 per cent. at 3.2. Powers ('23) studied the effect of carbon dioxide tension on the oxygen consumption of the herring. He found the maximum oxygen consumption at pH 7.6 to 7.8; alterations in the carbon dioxide tension, and hence pH, either above or below this value, lead to a decrease, rather slight, however, in the amount of oxygen consumed by the fish. Powers ('22) is of the opinion that the ability of fish to utilize oxygen depends to some extent on the pH of the water in which they are living.

In addition to such direct measurements of the effect of increased acidity of the medium on respiratory exchange there is available a considerable number of researches on the action of acids on processes in which respiratory metabolism is undoubtedly of great importance. Acidification of the medium has a general retarding or depressing effect on such processes. I can refer here only briefly to this literature. It is a well known fact, attested by many researches, that acidification of the medium retards the cleavage and development of the egg and produces abnormal types of larvæ (cf. Loeb, '98, Moore, Roaf, and Whitley, '05, Child, '16, Medes, '17, and Smith and Clowes, '24, for echinoderm eggs; Child, '25, for hydroids; Child, '17, for annelids; Loeb, '98, and Hall, quoted by Shelford, '23, for teleost eggs; and Hall, '24, and Bellamy, '19 and '22, on amphibian development). The retarding effect of acids on regeneration was noted by Jewell ('20). Acidification of the medium constitutes a general method for altering and controlling polarities (cf. Child, '23, and Rustia, '25).

For a general summary of the rôle of acids in the behavior and life of aquatic organisms the excellent paper of Shelford ('23) should be consulted. I am unable however to agree with Shelford that hydrogen ion concentration is of greater importance

than carbon dioxide content for aquatic organisms. The facts that Jewell ('22) found an extensive and varied fauna in an acid stream (pH 5.8 to 7.1) and Jewell and Brown ('24), several fishes living in an acid lake (pH 4.4) indicate that hydrogen ion concentration is not of paramount importance in the distribution of aquatic animals. Allee ('23) noted a lack of relation of hydrogen ion concentration to the distribution of marine animals.

From the investigations cited the general conclusion is certainly justifiable that the acidification of the medium to the proper extent retards or depresses biological processes and activities. My own experiments are in agreement with this general result. But without further analysis the conclusion should not be drawn that the observed effect is assignable to the free hydrogen ions of the acidified medium. Natural waters usually contain salts which undergo chemical changes when the water is acidified. In particular, carbon dioxide is evolved. Unless it is definitely proved that the evolution of carbon dioxide or other secondary chemical changes attendant on acidification of natural waters is not concerned in the result, the observed effect should not be assigned to the hydrogen ion concentration *per se*. Even ordinary distilled water contains some carbonates and other salts which may affect the result. Some of the researches cited in the foregoing review cannot be criticized on this score, or only to a slight extent, as distilled water was used, but in other cases, where natural waters were employed, the carbon dioxide evolved was probably of more consequence in the result observed than the hydrogen ion concentration.

In an extensive study of the depressing effect of acidification of natural waters on the oxygen consumption of aquatic animals, presented in this paper, it is shown that in all probability, carbon dioxide is chiefly responsible for the effect.

Methods.

The general course of procedure in the experiments to be reported was as follows. The rate of oxygen consumption of the animals in normal water was determined for a definite period of time, generally one hour. The normal water was then acidified to the desired extent and the oxygen consumption in

this acidified water for one or more successive periods of time determined. The animals were placed in Erlenmeyer flasks, if small, or in wide-mouthed salt bottles, if large, of about 500 cc. capacity. The water to be used was thoroughly aërated and placed in a large elevated receptacle. From this it was siphoned into the flasks or bottles containing the animals and allowed to flow out at the top for a few minutes. The flasks or bottles were then tightly closed and kept in a water bath at constant temperature for the desired length of time, generally one hour. After thoroughly shaking the contents, a sample of 125–150 cc. was then drawn by siphon and analyzed for oxygen content by Winkler's method. Blanks of the water used were of course also drawn at the beginning of the experiment and allowed to stand in the water bath with the animals until the end of the test. The difference between the oxygen content of these blanks and the samples drawn from the flasks or bottles containing the animals gives, after suitable calculation, the amount of oxygen consumed by the animals in the time selected. Immediately after drawing the normal samples, the acidified water was added in the same way and the animals allowed to respire in the acidified water for the same length of time. This was repeated as many times as desired, using the same animals and the same degree of acidification in any one series of experiments. The water was freshly acidified and aërated for each of these determinations. There is no possibility that alterations in the oxygen content of the water in any way affected the result, as the oxygen content was at saturation at the beginning of each test, and the test continued only long enough for the animals to use up a small part of the oxygen present.

The hydrogen ion concentration was determined by means of indicators purchased from the La Motte Chemical Products Company. A set of standard tubes covering the range from pH 3.0 to pH 8.4, with indicators brom phenol blue, brom cresol green, brom cresol purple, and phenol red, was employed. Solutions of these indicators were obtained from the same company. A few drops of the appropriate indicator added to 10 cc. of the water to be tested yields a color which can be compared with that of the standard tubes; the approximate pH is thus

easily obtained. Brom cresol green was not found to be reliable and consequently pH 5.0 could not be determined very exactly.

The acids employed were: hydrochloric, nitric, sulphuric, carbonic, acetic, butryic, citric, and tartaric. Each of these acids was used in the following concentrations, in terms of pH: 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, and in some cases 4.5. At least three experiments at each concentration of each acid were performed and in many cases a greater number of trials was deemed necessary. It will be seen that the amount of labor involved was very great and this fact must serve as my excuse for any deficiencies in the investigation which may suggest themselves to the reader.

Experiments with Marine Animals.

The experiments recorded in this section were performed in 1922 at the Hopkins Marine Station, Pacific Grove, California. The animals used were two species of starfish, *Leptasterias equalis*, and *Patiria miniata*, and the nudibranch, *Anisodoris nobilis*. It may be noted in passing that starfish and nudibranchs have been found to constitute very favorable material for respiratory experiments.

The animals were placed in wide-mouthed salt bottles and the tests carried out as outlined above. *Leptasterias* is a small species and several individuals were therefore used in each bottle. As the other two species are larger, generally only one individual was necessary. Generally two successive trials of the oxygen consumption in normal sea-water were carried out. The sea-water was then acidified to the desired amount with hydrochloric acid and a determination immediately carried out. The animals were then usually allowed to stand for two to several hours in the acidified sea-water; another test was then performed. The animals then remained in the acid water over night, about 13 to 15 hours in different experiments, whereupon a third test of the respiration in the acidified water was performed. The animals were then returned to normal sea-water, and after several hours the oxygen consumption was again tested in normal sea-water.

It should be understood that for each test freshly aërated and freshly acidified water was used. The water was brought in directly from the ocean as the sea-water coming through the

pipes into the laboratory was found to be toxic. In acidifying the water, concentrated HCl was regarded as a 10 mol. solution and sufficient of this was added to a measured quantity of sea-water to give theoretically a certain molecular concentration of hydrochloric acid. Actually, of course, owing to the salts in the sea-water, the concentration obtained is less than that given in the tables. The hydrogen ion concentrations of these solutions could not be determined as I did not have with me the necessary outfit. It may be roughly estimated, however, that 1/1000 HCl in sea-water is about pH 7.0, 1/800 about pH 6.5, 1/600 about pH 6.0, and 1/400 between 5.0 and 5.5. When allowed to stand, of course, such solutions become continuously more alkaline, owing to the escape of carbon dioxide. For this reason, the solution was not made until a few minutes before it was to be used in the tests. The pH of normal sea-water is about 8.2.

The acidification of the sea-water has little or no noticeable effect upon the behavior of the animals in the lower concentrations, 1/1000 mol. or less. In concentrations of 1/800 mol. or greater, the animals are markedly affected. The starfish withdraw their tube feet into the ambulacral groove. The rays are retroflexed towards the aboral surface. In this position the animals remain during the exposure to acid and are apparently unable to move about or to cling with the tube feet. Nudibranchs display a similar behavior. In the stronger solutions they lose their ability to hold to the substratum with the foot and float about in the water in a state of immobility. The gills however remain expanded and it is not believed that differences in the degree of expansion of these organs are to any extent responsible for the depressing effect of acid on the oxygen consumption. In brief, acidification of the water appears to paralyze the neuro-muscular apparatus and to reduce the animal to a state of forced immobility. Recovery is prompt and complete upon return to normal sea-water. None of the concentrations used had any injurious effects upon the animals.

The results of the experiments are given in part in Tables I and II. A typical experiment for each concentration of acid used for one of the starfish species (*Patiria miniata*) and for the nudibranch is presented in Table I. Three trials of each concen-

TABLE I.

EFFECT OF THE ACIDIFICATION OF THE SEA-WATER WITH HYDROCHLORIC ACID ON THE RATE OF OXYGEN CONSUMPTION OF THE STARFISH *Patiria miniata*, AND THE NUDIBRANCH *Anisodoris nobilis*.

Oxygen consumed given in cubic centimeters, per ninety minutes for the starfish, per hour for the nudibranch. Each vertical column constitutes one experiment, showing successive tests of the oxygen consumption of the same animals or animal. The concentration of the acid is given in molecular strengths.

Starfish.				Nudibranch.			
Oxygen Consumption in Normal Sea-water.							
.43	.40	.31	.34	.78	1.02	.68	.83
Oxygen Consumption in Sea-water Acidified with HCl.							
1/1000	1/800	1/600	1/400	1/1500	1/1000	1/800	1/600
Immediate Effect.							
.41	.32	.23	.22	.76	.77	.49	.50
Effect after 2 to 6 Hours' Exposure.							
.35	.31	.21	.22	.74	.78	.57	
Effect after 13 to 15 Hours' Exposure.							
.42	.34			.74	.75		
Recovery on Return to Normal Sea-water.							
.42	.44	.32	.34	.76	.97	.72	
Initial per cent. Depression.							
5	20	26	36	3	25	28	40

TABLE II.

AVERAGE INITIAL PER CENT. DEPRESSION OF ALL EXPERIMENTS ON MARINE ANIMALS.

Conc. HCl.	1/1500	1/1000	1/800	1/600	1/400
Leptasterias		31	38	38	51
Patiria		9	21	28	34
Anisodoris	7	30	26	31	

tration were made but only one of these is given in the table, and may be taken as typical of the three. The results on the other species of starfish (*Leptasterias equalis*) being in no wise different from those on Patiria are omitted for the sake of economy of space. In Table II. is given a summary of all of the experiments, the three trials with each concentration being

averaged. The percentage of depression is figured from the oxygen consumption during the first exposure to the acid.

These experiments, although somewhat preliminary in nature, nevertheless exemplify the action of acids on respiratory metabolism. The conclusions which they furnish were verified by later more extensive work. These conclusions may be briefly stated at this point:

1. Acidification of the medium lowers the rate of oxygen consumption of animals.

2. This depression is greater the greater the degree of acidification up to a certain point.

3. Low concentrations are, however, relatively more effective than high concentrations.

4. After a certain degree of acidification has been attained, further acidification does not greatly increase the amount of depression of the oxygen consumption. The maximum depression obtainable is about 50 per cent.

5. The depressing effect of acids on respiratory metabolism is completely reversible, providing the concentration of acid employed is not actually injurious to the tissues of the animal.

Experiments with *Planaria* in Unaltered Fresh Water.

The experiments to be reported in the remainder of the paper were performed at Chicago and occupied the greater part of my time from October, 1923, to February, 1925. The aim of the experiments was to test the effect upon the oxygen consumption of a fresh water animal of the acidification of fresh water with various acids at a variety of hydrogen ion concentrations. The animal selected for this purpose was the flatworm, *Planaria dorotocephala*. It was chosen for a variety of reasons: it is very abundant, is easily kept under laboratory conditions, has no calcareous parts which might be affected by acid, can be kept quiet during respiratory work, and finally had already served as material for a large amount of physiological experiment. I had originally intended to work also with fish and crayfish but the experiments on *Planaria* required a much greater expenditure of time than I had anticipated and I did not feel justified in spending any more time on the matter. I was further informed

by Dr. E. B. Powers that he was already engaged in a similar investigation on fish, and I was quite willing to relinquish this particular task to him.

1. *General Procedure.*—There is little to add to the procedure already outlined. Large stocks of *Planaria dorotocephala* are always at hand in our laboratory. Only stocks which had sojourned in the laboratory for at least one or two months were employed, as the basal respiratory rate of freshly collected material is variable. Only worms which had starved from four days to two weeks were used, since during this period the respiratory rate is constant. Each lot of worms to be used for an experiment consisted of a number of individuals sufficient to consume a readily measurable amount of oxygen in an hour. Such a lot was placed in a 500 cc. Erlenmeyer flask at least several hours before the experiment because when the worms are placed in any clean strange container they are apt to travel about restlessly for some time. By placing the worms in advance in the container in which they are to be tested and by darkening them during the experiment, movement can be practically entirely eliminated. It is certain that movement does not play any rôle in the results. Three such lots of worms were carried throughout the work in order that three tests of each strength of each acid could be performed simultaneously. This procedure shortens considerably the amount of labor involved. Each lot of worms was kept in a particular flask for nearly two weeks and used several times during that period. If desired such lots could then be fed once or twice in the flasks and after four days again utilized for experiments. This procedure was found most suitable because different lots of worms are apt to yield different percentages of depression with the same concentration of the same acid. To obtain a graded set of results showing a graded increase in the percentage of depression with increasing concentration of acid, it is almost necessary to test all of the concentrations of any one acid on the same lot of worms. If different lots are interpolated in such a series, the relation between depression and concentration is less regular, although always exhibiting the same general trend.

The remainder of the procedure was as already described. It

may be repeated once more that for each hour's test freshly aërated water was employed and this was freshly acidified to the desired degree a few minutes before being used for each hour's determination. Each test lasted for one hour. A determination of the normal rate of oxygen consumption in normal water (pH 8.0) was first run, and this was followed without pause by a determination in the acidified water. In many cases there were two or three trials in the acidified water, usually with intervals between the trials. During such intervals the animals remained of course in the acidified water. As it was found by repeated experiment that the maximum percentage of depression is obtained on the initial exposure to the acid, these later tests in acid were omitted in the latter part of the investigation. Generally the investigation of each acid was begun with the lowest concentration (pH 7.5) and the concentration increased by steps of 0.5 pH at each succeeding experiment. As will be discussed later this procedure gives the most regular results and great irregularities are introduced if the strongest concentrations are employed first.

There is not the slightest possibility that any differences in the oxygen content of the water either at the start of each test or produced during the test by the withdrawal from the water of oxygen by the animals in any way affects the results. The oxygen consumption of *Planaria dorotocephala* is entirely independent of the oxygen content of the water at all concentrations between 8 and 2 cc. per liter. Whether it is affected by concentrations above or below these limits has not been determined. Suffice it to say that in none of the experiments here recorded nor in any that I have reported in the past with this species has the oxygen content of the water ever reached a value which could have affected to the slightest degree the rate of oxygen consumption of the animals.

The acids used and the hydrogen ion concentrations at which they were tested have already been stated. Concentrations which might have injured the animals during the exposure were avoided. For this reason very few tests were run at acidities greater than pH 5.0. It was desired to keep within physiological concentrations.

All of the experiments on *Planaria* were performed at a temperature of 20° C. and the oxygen in all cases is calculated for this temperature. The results are given in cubic centimeters of oxygen consumed per hour. The animals were not weighed as the oxygen consumed per gram is of no importance for the present experiments. For the benefit of those who may be curious regarding this matter it may be stated that the normal rate of oxygen consumption of this species under the conditions of the experiments (worms of medium size, four to fourteen days after feeding, temperature 20° C.) is about .20 cc. of oxygen per gram per hour.

2. *Water.*—The water used in the experiments comes from a well driven into the ground from the floor of the basement of the laboratory. This water when it emerges from the taps is supersaturated with air and also contains a considerable amount of free carbon dioxide. It has a pH of about 7.3. On account of the gas content the water is heated but not boiled and then allowed to cool overnight before being used for experimental purposes. Water so treated has a pH of 8.0 to 8.2. This is the water regarded in the experiments as "normal" and the respiration in such water is taken as "normal." The water is of course thoroughly aërated before use, bringing the oxygen content up to 6 to 8 cc. per liter.

TABLE III.

ANALYSIS OF THE WATER USED IN THE EXPERIMENTS ON *Planaria*.

All figures represent parts per million. Analysis made in 1922.

Determinations as		Hypothetical combinations.	
Iron—Fe	0.2	Potassium nitrate	1.4
Manganese—Mn	0.1	Potassium chloride	1.1
Silica—SiO_2	7.8	Sodium chloride	7.6
Nonvolatile	0.7	Ammonium chloride	0.1
Alumina—Al_2O_3	1.6	Magnesium chloride	1.0
Calcium—Ca	49.2	Magnesium sulphate	22.1
Magnesium—Mg	16.7	Magnesium carbonate	41.5
Ammonia—NH_4	0.03	Calcium carbonate	123.0
Sodium—Na	3.0	Silica—SiO_2	7.8
Potassium—K	1.1	Alumina—Al_2O_3	1.6
Sulphate—SO_4	17.7	Iron oxide—Fe_2O_3	0.3
Nitrate—NO_3	0.9		
Chloride—Cl	6.0		
Alkalinity (methyl orange)	170		
Residue	193		

The water used is thus the ground water of the Chicago region. It is high in salt content, particularly in carbonates. This water has been analyzed for us by the State Water Survey Division of Illinois. It seems desirable to present the analysis at this point.

3. *Behavior of Planaria in Acid.*—When the water is acidified no alteration in the behavior of the worms is noticeable until a certain degree of acidification is reached. This degree is different for different acids, but is generally in the neighborhood of pH 5.5 to 5.0. The animals lose the ability to glide about, owing apparently to a paralysis of the ciliary mechanism. They exhibit continuous writhing movements, and secrete a large amount of mucus. With greater acidification, they lose ability to cling to the glass and fall to the bottom of the container, where they remain, often with slight writhing movements. They are, when this condition is attained, extremely elongated and generally more or less curved, the ventral surface being concave, the dorsal convex. In short, as in the case of the marine animals, a sufficient degree of acidity induces a paralysis of the motor mechanism, which appears to involve the cilia first, the muscles later.

4. *Experiments with Hydrochloric Acid.*—A large number of experiments, about seventy, were performed with this acid. The normal respiration in normal water (pH 8.0) for one hour was first determined. The water was then acidified to the desired degree and the rate of oxygen consumption in the acid water immediately tested. An interval of one hour in the acidified water was then passed, another determination made in freshly acidified water, another hour passed, and a third determination carried out. It will thus be evident that the respiration during the first, third, and fifth hours in the acid was tested. This was the general procedure in the earlier part of the work. The acidity ranged from pH 7.5 to 4.5 at 0.5 pH intervals. It seems unnecessary to present in detail the mass of data thus accumulated as all of the experiments gave the same general result. A set of typical determinations of the action of hydrochloric acid at different hydrogen ion concentrations is given in Table IV. The averages of all of the experiments performed with this acid are summarized in Table VI. The action is graphed in Fig. 1.

TABLE IV.

ACTION OF HYDROCHLORIC AND SULPHURIC ACIDS AT DIFFERENT HYDROGEN ION CONCENTRATIONS ON THE OXYGEN CONSUMPTION OF *Planaria*.

All figures represent cc. of oxygen consumed per hour at 20° C. Each vertical column constitutes one experiment, giving successive determinations on the same lot of worms.

Normal Respiration, pH 8.0.													
	.42	.44	.37	.48	.34	.34	.42	.43	.38	.40	.50	.58	.43
Respiration in Acidified Water.													
	Hydrochloric.							Sulphuric.					
pH	7.5	7.0	6.5	6.0	5.5	5.0	4.5	7.5	7.0	6.5	6.0	5.5	5.0
1st hr.	.36	.34	.28	.34	.23	.20	.23	.36	.27	.27	.33	.37	.24
3d hr.	.38	.37	.33	.37	.24	.22	.25	.38	.30	.34	.39		
5th hr.	.38	.33	.30	.38	.27	.20		.34	.34	.26	.43		
Per cent. Initial Depression.													
	13	23	25	29	33	40	46	16	29	35	34	37	45
Recovery after Return to Normal Water.													
	.44	.41	.41	.41	.34	.32	.35	.49	.39	.41	.44	.49	.41

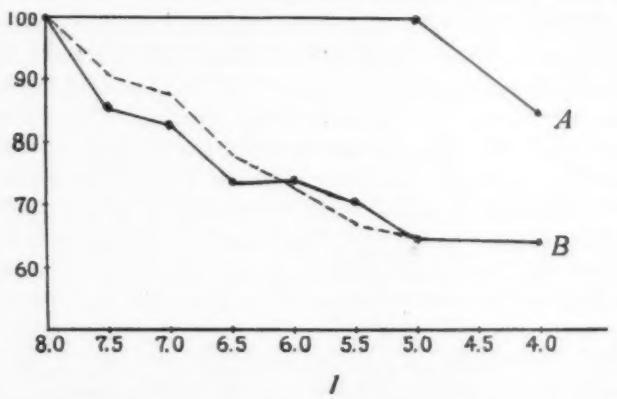

FIG. 1. Graph of the action of acids on the rate of oxygen consumption. Curve *A*, action of hydrochloric acid in carbonate-free water. Curve *B*, action of hydrochloric acid in ordinary water. Dashed line, average curve of the depressing action, obtained by averaging all of the acids except butyric. Percentage of depression, normal respiration being taken as 100, on the ordinate, pH on the abscissa.

It will be perceived by examining these tables that the rate of oxygen consumption of *Planaria* is decreased by acidifying the water with hydrochloric acid and that the percentage of depression is greater the higher the acidity. Yet it must be remarked at this point that the percentage of depression obtained with any given concentration of acid is subject to inexplicable variation even on the same lot of worms and quite commonly when different lots of worms are employed. In most cases this variation did not exceed 10 per cent. but it may reach 20 to 30 per cent., in the higher concentrations. On account of these puzzling variations I was frequently compelled to repeat the experiments many more times than had been anticipated as necessary. The matter of variation is considered at more length later and a possible cause is suggested.

TABLE V.

ACTION OF CARBONIC AND ACETIC ACIDS AT DIFFERENT HYDROGEN ION CONCENTRATIONS ON THE OXYGEN CONSUMPTION OF *Planaria*.

All figures represent cc. of oxygen consumed per hour at 20° C. Each vertical column constitutes one experiment, giving successive determinations on the same lot of worms.

Normal Respiration, pH 8.0.												
	.34	.21	.31	.29	.28	.25	.40	.50	.50	.36	.51	.71
Respiration in Acidified Water.												
	Acetic Acid.						Carbonic Acid.					
pH	7.5	7.0	6.5	6.0	5.5	5.0	7.5	7.0	6.5	6.0	5.5	5.2
1st hr.	.30	.26	.28	.24	.20	.14	.44	.46	.39	.27	.35	.43
3d hr.	.36	.26	.37	.28	.25	.14		.45		.25		
5th hr.	.31		.32									
Per cent. Initial Depression.												
	12	+23	10	18	29	44	+10	8	22	25	32	40
Recovery after Return to Normal Water.												
	.36	.20	.29	.28	.31		.41	.50	.46	.44	.52	

5. *Experiments with Sulphuric and Nitric Acids.*—The action of these two mineral acids was very similar to that of hydrochloric acid. The percentage of depression induced was consistently slightly greater at the same hydrogen ion concentrations

than that produced by HCl. Some detailed data on sulphuric acid are given in Table IV. and the results of all experiments with nitric and sulphuric acids are summarized in the general table, Table VI.

TABLE VI.

SUMMARY OF ALL EXPERIMENTS WITH ALL ACIDS, GIVING THE AVERAGE PERCENTAGE OF DEPRESSION ON INITIAL EXPOSURE TO EACH ACID AT EACH pH USED.

pH.	7.5	7.0	6.5	6.0	5.5	5.0	4.5	3.8
Hydrochloric	14	17	26	26	29	35	45	36
Sulphuric	21	22	27	33	33	34		
Nitric	18	26	28	30	40	40		
Carbonic	+ 2	3	20	21	31	45		
Acetic	6	+ 8	10	31	30	40[1]		
Butyric	16	7	15	7	8	37[1]		
Tartaric	13	14	22	21	31	33		
Citric	+ 4	10	18	31	40	29		

6. *Experiments with Carbonic Acid.*—Carbon dioxide gas was passed through the water until the desired acidity was obtained. In this procedure great care was necessary to avoid depriving the water of its oxygen, since when a gas bubbles through a liquid it removes other gases. The water had to be vigorously shaken with air after the carbon dioxide had been added. A good deal of manipulation is required when working with higher concentrations of carbon dioxide to secure the proper amount of oxygen and carbon dioxide simultaneously in the water. In all of the experiments reported the oxygen content of the water was ample. It may be noted in passing that a combination of low oxygen with high carbon dioxide has a much greater depressing effect than either of these conditions separately. Under ordinary conditions the rate of oxygen consumption is normal in water containing 2 cc. per liter but oxygen consumption is almost abolished in such water if it be saturated with carbon dioxide. When plenty of oxygen is present, the depression produced by saturated carbon dioxide is only about 50 per cent.

Most of the experiments with carbon dioxide are reported in Table V. In the more dilute solutions—pH 7.5 to 7.0—carbon dioxide acidity produces less depression than do other mineral

[1] Animals injured.

acids. In fact, in these concentrations, there is a tendency towards stimulation of the respiratory rate. Out of nine experiments, stimulations of 2 to 12 per cent. were obtained in five cases. The amount of stimulation, however, lies within the experimental variation. The truth probably is that carbon dioxide acidity does not affect the rate of oxygen consumption until the acidity reaches a greater degree than pH 7.0. At greater acidities than pH 7.0, the rate of oxygen consumption is depressed and to a degree similar to that produced by other mineral acids. It was not possible to obtain an acidity with carbon dioxide greater than pH 5.2 with the well water, owing probably to the buffers present in the water.

7. *Experiments with Acetic and Butyric Acids.*—These two acids were selected as examples of the paraffin acids, supposed to penetrate organisms readily. It was anticipated that the depressing effect of these acids would be greater than that of mineral acids. Such, however, was not the case, but the contrary result appeared. Acetic acid, in acidities between 7.8 and 7.0 tended to stimulate the rate of oxygen consumption. Of six experiments performed at pH 7.5, the rate of oxygen consumption was unaffected in two cases, stimulated in two, and depressed in the remaining two. Of six experiments at pH 7.0, all but one were stimulating. It seems probable that low concentrations of acetic acid accelerate the rate of oxygen consumption. At concentrations greater than pH 7.0, all concentrations depressed the rate of oxygen consumption during the first hour's exposure. During later exposures, a tendency toward acceleration was again manifest. These facts are brought out in Table V. and VI. Butyric acid was the least effective in inducing depression of all of the acids used. This result was very surprising and still remains inexplicable. Butyric acid has only a slight depressing action at all concentrations between pH 7.5 and 5.5. At pH 5.0 a depression of 30 to 40 per cent. appears but this concentration of butyric acid is lethal and the worms begin to die within an hour. Acetic acid at pH 5.0 is also injurious. The data on butyric acid are summarized in Table VI., which gives the initial depression induced. Later exposures did not alter the result.

8. *Experiments with Tartaric and Citric Acids.*—The former was

selected as an example of a dibasic, the latter of a tribasic acid. The results were not of particular interest and are summarized in the general table, Table VI. The action of tartaric acid was very similar to that of the mineral acids. Citric acid was less effective than the inorganic acids at concentrations lower than pH 7.0.

9. *Consideration of Other Factors.*—(*a*) *Size.* It was found long ago by Child that young (small) individuals are more susceptible to lethal concentrations of various substances than are old (large) individuals of a given species. MacArthur ('20) studied the relation between age (size) and susceptibility to acids in *Planaria dorotocephala*. He found that in concentrations of hydrochloric and other acids which are quickly lethal, between pH 2.0 and 4.5, the young (small) individuals die sooner than the old (large) ones. At slightly lower concentrations, pH 4.7 to 4.9 for HCl, the relation is reversed, the larger individuals succumbing first. In still weaker solutions, planarians live indefinitely. These results have been repeatedly confirmed in this laboratory in class work, although of course the precise concentrations required to yield the results mentioned are subject to variation, owing chiefly to differences in the physiological condition of the worms themselves.

Since according to these results, small worms are more susceptible to acids than large worms at concentrations greater than pH 4.5, it seems probable that the percentage of depression induced by acids should bear some relation to size. This was tested in a number of experiments, in which the depressing action of HCl on large and on small worms was compared. Only recently collected material was used as the metabolic differences between worms of different sizes are greater in such material. The heads of all worms were removed before the test, in order to eliminate movement, as small worms are more active than large ones. Decapitated worms are very inactive. The small worms used were under 10 mm. in length, the large ones over 20 mm. Twelve experiments were performed at concentrations of HCl ranging from pH 3.6 to 4.6. In ten of these the percentage of depression was greater in the small than in the large worms. The difference ranged from 3 to 20 per cent. In two cases the

result was reversed, the larger worms exhibiting about 10 per cent. more depression than the small ones. In general, then, the experiments indicate that in concentrations which will kill within a few hours and in which small worms die slightly faster than large worms, the small worms are more greatly depressed by the acid. It should be added that the animals suffered no injury during the period of exposure and recovered completely.

(*b*) *Previous History with Respect to Acid.*—From my experiments I have gained the distinct impression that the amount of depression induced by exposure to acidified water is to some extent dependent on the previous history with respect to acid. If a given lot of worms, which has never been exposed to acidified water, is tested at rather high acidity, say pH 5.0, it is commonly found that the amount of depression is much less than would ordinarily be expected at that acidity. If on the other hand, such worms are gradually accustomed to acidified water, by exposing them first to pH 7.5 and gradually increasing the acidity, then a much greater percentage of depression is obtained at high acidities. One of the most striking cases of this kind in my investigation occurred in working with sulphuric acid. Three lots of worms, never before exposed to acidified water, were tested in water acidified to pH 5.0 with sulphuric acid. The percentages of depression obtained were 20, 30, and 15 respectively. The same lots of worms were then exposed on successive days to various concentrations of sulphuric and hydrochloric acid, beginning at 7.5 and gradually working down to pH 5.0 again. When sulphuric acid pH 5.0 was then again tried, on the same worms, the depressions obtained were respectively 39, 56, and 45 per cent. The same type of result was frequently met with. It appears that when worms are suddenly exposed to rather strongly acidified water, they are able to produce or manufacture some substance which protects them from the acid. But after repeated exposures to acidified water, the substance is either exhausted or the worms have become so accustomed to residing in acidified water that they no longer respond to it by producing the substance. Sudden exposure to acid may act as a stimulus to production of basic substances; repeated exposure fails to stimulate.

It seems probable that in this behavior of the worms is to be found the explanation of the numerous puzzling variations in the degree of depression obtained.

(*c*) *Acclimation.*—It was my intention at the beginning of this work to determine whether the animals could recover from the depression induced by acid if allowed to remain for some time in the acidified solution. I found, however, that the experiment is impractical, because of the carbonate content of the water used. When the water is acidified it soon becomes alkaline and unless acidified to a point which would be fatal to the worms, returns to an alkaline condition within 24 hours. Consequently if worms are placed in the acidified water at a certain pH, the water does not remain at this pH, but the pH rises (the acidity falls). If then the worms are tested at the new pH, the depression is naturally less than it was at the beginning of the exposure. If tested at the original pH, the depression is greater than at the pH attained by the standing solution. In brief, it is impossible to determine the effect of long continued exposure to a given pH, unless the water is freshly acidified and changed every hour, for two or three days. As this is physically impossible, for me at least, the experiment had to be abandoned. I have, however, a number of experiments, especially with HCl, in which the oxygen consumption was tested during the first, third, and fifth hours of continuous exposure to a given concentration. Some of these data appear in Table IV. In general it was found that there is very slight if any recovery during successive hours of exposure. After this had been repeatedly determined, the tests of the later exposures were abandoned.

The reader will no doubt at once inquire why carbonate-free water was not employed in a study of acclimation. The reason for this as will appear later is that acidified carbonate-free water does not affect the oxygen consumption of Planaria.

10. *General Results and Discussion.*—We may now state and discuss the results obtained with acidified unaltered water.

(*a*) Acidification of the water generally causes a depression of the rate of oxygen consumption. This was true of the three mineral acids, hydrochloric, sulphuric, and nitric, at all concentrations employed. Even a change from the normal pH 8.0 of

the water to 7.8 by addition of such acids induces a measurable lowering of the rate of oxygen consumption. In the case of some acids, notably acetic, less so with carbonic, there was some tendency to an acceleration or stimulation of the oxygen consumption at acidities between 7.8 and 7.0. Yet this effect was so slight, and the variability of the results so great that little emphasis can be placed upon this finding, unless a very large number of experiments were carried out. All of the acids used caused depression at all acidities greater than pH 7.0, but the action of butyric was very slight, almost nil.

(*b*) The percentage of depression increases with increasing acidity within certain limits.

(*c*) The lower concentrations are, however, relatively more effective than the higher concentrations. This is generally the case wherever chemicals are applied to living organisms. The general form of the curve obtained is seen in Fig. 1. This type of curve is so commonly obtained in physiological experimentation that it must possess some deep significance. I am unable however to suggest any explanation of this type of curve.

(*d*) The inorganic acids, except carbonic, are in general more effective than the organic, particularly in the lower concentrations, pH 7.5 to 6.5. This finding was the contrary of my expectations and contrary to the results of Gray ('24). It is generally believed that organic acids, such as acetic and butyric, penetrate protoplasm readily, while mineral acids are unable to do so. Obviously one would then expect the organic acids to act more powerfully on respiratory metabolism than the inorganic acids. As this was not found to be the case, it must be concluded that the penetrability of the acid has no bearing on the result. The lack of action of butyric acid remains inexplicable.

(*e*) The hydrogen ion concentration cannot be the chief cause of the depression induced, because different acids do not produce the same percentage of depression at the same pH. The result does not substantiate the contentions of Loeb ('22) that the action of acids on colloids depends only on valence and hydrogen ion concentration. According to Loeb's ideas, all of the monovalent acids, such as hydrochloric, nitric, acetic, and butyric

should have produced the same amount of depression at the same hydrogen ion concentration. Reference to Table VI. shows that this is not at all the case, butyric acid furnishing a notable exception. According to Loeb also, di- and tri-basic acids should be less effective than monobasic. This again is not upheld by my results. Sulphuric acid is even more effective than hydrochloric, although being dibasic it should be only half as effective. Tartaric, another dibasic acid, has about the same efficiency as the monobasic mineral acids. Of course, it is not certain that Loeb intended his ideas to apply to living organisms. Further the hydrogen ion concentrations with which Loeb worked are mostly instantly or rapidly fatal to living organisms. It remains to be proved whether the statements of Loeb will hold at physiological concentrations of acid. I have been informed that Michaelis has publicly stated that they do not hold and that the Hofmeister series remains unshaken. From the fact that in my experiments different acids produce different degrees of depression at the same hydrogen ion concentration it appears necessary to conclude that the hydrogen ion concentration is not the principal factor in the result.

(*f*) When a certain degree of acidification has been attained, further acidification does not increase the percentage of depression. The percentage of depression obtained at pH 5.0 is about the maximum that can be produced without actual injury to the animals. It appears that a depression of 50 per cent. is the most that can be obtained with any acid on the average. Of course individual experiments may yield a depression slightly greater than this. A depression of 58 per cent. is the greatest recorded in the dozens of experiments performed with the various acids and this figure was obtained on young worms. The statements in this paragraph apply only when an ample supply of oxygen is present in the water.

(*g*) The decrease in the rate of oxygen consumption reaches its maximum value for any particular concentration during the first hour of exposure to the acidified water. Prolonged exposure does not increase the depressing effect. On the contrary there is generally some slight rise in the oxygen consumption during several hours exposure. This is too small however to be considered of significance.

(*h*) The depressing action of acids is completely and promptly reversible, wherever an actual injury to the tissues of the animal has been avoided. Recovery occurs almost immediately, commonly within the first hour after return to normal water. Several experiments were devised to test the possibility that the decreased oxygen consumption while in the acid might be compensated for by an increase over the normal during the period immediately upon return to normal water. In these experiments the oxygen consumption was first tested in normal water, then in acidified water, of a concentration to give at least 30 per cent. depression, then immediately in normal water again. In most of these cases the respiration had risen to the normal value during the first hour after return to normal water. In a few cases, the oxygen consumption was below normal. In no case was any rise over the normal figure observed.

(*i*) The experiments justify the use of acids as agents for experimentally producing a state of depression. They also substantiate the generally held view that the effects produced by acids on such processes as cleavage, development, and regeneration are assignable to a reduction in the rate of respiratory metabolism.

Experiments with *Planaria* in Carbonate-free Fresh Water.

The experiments had reached the point outlined above by the summer of 1924 and I intended to bring the investigation to a close. A number of matters puzzled me greatly but I was unable to devise any means of throwing further light on them. At about this time, however, my attention was drawn to the experiments of Clowes and his associates ('23, '24) in which it was shown that the carbon dioxide set free by acidification of sea-water markedly influences the result and is in some cases the real agent involved. At first I was not inclined to believe that carbon dioxide was responsible for the results which I had obtained with acids. It seemed to me that if carbon dioxide were chiefly or wholly responsible for the observed effects, carbon dioxide acidity should be more effective than acidities produced by other acids and the action of various acids should be similar

at the same hydrogen ion concentrations. As these conditions did not obtain in my experiments, I considered it unlikely that the findings of Clowes applied to them. It seemed necessary to me, however, that the matter should be tested by experiment. A new series of experiments was therefore begun in the fall of 1924 using carbonate-free water. The results obtained convinced me that the depressing action of acidified water is largely due to the carbon dioxide liberated in it.

In connection with these experiments a large number of trials in ordinary carbonate-containing water were carried out as controls. These constituted repetitions of the work of the previous year. It was very puzzling to me to find that all acids were less effective than had been the case in the preceding year. This was particularly noticeable at the lower concentrations, pH 7.5 to 7.0. The mineral acids produced about the same depressing effect as previously at the higher concentrations, 6.5 to 5.0. The organic acids (acetic and butyric were the only ones tried) were also markedly less effective than had previously been the case, at all concentrations tried. I am unable to explain this state of affairs except on the assumption that the carbonate content of the water had decreased in the meantime. The fact that in many cases the controls gave a smaller percentage of depression than previously made it difficult to arrive at conclusive results.

1. *Preparation of Carbonate-free Water.*—Carbonate-free water was prepared according to the method of Smith and Clowes ('24*b*). Two cc. of concentrated hydrochloric acid were added to eight liters of the well water in a large bottle. Air from the compressed air system was passed through the water in the bottle for 24 hours or more. This treatment was found to remove the carbonate from the water completely. Several times liter samples of this water were evaporated to dryness and the residue tested for carbonate with entirely negative results. In some cases borax was added to the carbonate-free water to serve as buffer but as the results were not affected by this procedure, it was generally omitted. The oxygen consumption of the worms in this carbonate-free water, with or without borax, was repeatedly compared with that in the normal unaltered well water. No difference was found.

2. *Action of Mineral Acids Added to Carbonate-free Water on the Rate of Oxygen Consumption.*—The same amount of acid was added to the water as would be required to produce the desired pH in unaltered water. Alkali was then added until the pH in question had been attained. Rather extensive experiments were performed with hydrochloric acid, less extensive with sulphuric and nitric acids. The results in all three acids were the same and were very striking. Acidification of carbonate-free water with these acids has *absolutely no effect* on the rate of oxygen consumption of Planaria at all concentrations between pH 7.5 and 5.0. At pH 4.0, a slight depressing effect was noted, about 15 per cent. This result is graphed in Fig. 1.

It is thus proved that practically the entire depressing effect on the oxygen consumption of *Planaria* produced by the addition of mineral acids to natural water arises from the carbon dioxide set free in such water by decomposition of its contained carbonates by the acid added. The depressing effect is thus a carbon dioxide depression. It is probable that the carbon dioxide penetrates the animal in the gaseous state and acts within the animal as such or by inducing an internal acidity (cf. Jacobs, '20).

3. *Action of Organic Acids in Carbonate-free Water.*—Only acetic and butyric were tried. I was particularly interested in testing these two acids as they are believed to penetrate organisms readily. The result should serve to indicate whether the efficiency of carbon dioxide is merely a question of penetrability. Unfortunately decisive results could not be secured, owing to the fact, already mentioned, that very little depressing effect was obtained in the controls. Butyric acid, previously found to be the least effective of all of the acids tried, was in this series of experiments quite ineffective in normal water. Even at pH 5.0, an injurious concentration, no depression of the oxygen consumption in normal water appeared. Consequently the action of butyric acid in carbonate-free water could not be determined. With acetic acid, some results were secured. Acetic acid was about half as effective in inducing depression as in the experiments of the preceding year. At concentrations weaker than pH 7.0 there were again indications of acceleration of the rate of oxygen consumption in the controls. At acidities of pH 6.5

or greater, depression was produced in normal water, but to a less extent than previously. In the same worms, in carbonate-free water acidified with acetic acid, the depression induced was noticeably less than in the controls. It therefore follows that the depressing action of acetic acid is also in large part due to the carbon dioxide which is sets free. It appears that the penetrating powers of carbon dioxide do not entirely explain its difference from other acids.

4. *Action of Carbon Dioxide Added to Carbonate-free Water.*—These experiments were designed as a sort of crucial test of the proposition that carbon dioxide is the cause of the depression induced by acidification of natural waters. The proposition was upheld in the most striking manner. The addition of carbon dioxide gas to the same pH causes the same degree of depression, whether added to normal, or to carbonate-free water. It thus appears to be reasonably certain that the depression of the rate of oxygen consumption in acidified water is caused chiefly by carbon dioxide.

The question then arises: Is the amount of carbon dioxide liberated in carbonate-containing water by the addition thereto of acids, different with different acids at the same hydrogen ion concentration? It became necessary to determine the actual amount of free carbon dioxide present when the well water was acidified with various acids.

5. *Determinations of the Amount of Carbon Dioxide Liberated on Acidification of the Unaltered Water.*—The normal well water, pH 8.0, was acidified to the desired pH with various acids. A sample of 100 cc. was then immediately drawn and the free carbon dioxide in it determined by titration with $N/50$ barium hydroxide, properly protected from the carbon dioxide of the air, using phenolphthalein as indicator.

Hydrochloric, sulphuric, acetic, and butyric acids were investigated at ranges of pH 7.5 to 4.0. It was found that the amounts of carbon dioxide liberated from the carbonates of the water by different acids are similar, but not identical, at the same pH. Such differences probably account for some of the different percentages of depression obtained with different acids at the same pH. But butyric acid was also found to liberate nearly as

much carbon dioxide at the same pH as does hydrochloric acid. The lack of depressing action of butyric acid therefore remains at present inexplicable.

Nearly all of the carbonate of the water is liberated as carbon dioxide at a pH of 5.0 and all of it between pH 5.0 and 4.0. The amount of free carbon dioxide present at these acidities is about 30 cc. per liter. At first the fact that all of the available carbonate in the water is decomposed at pH 5.0 to 4.0 appeared to explain the result that the maximum percentage of depression is obtained at such acidity and further acidification does not increase the depression. But the additional fact discussed in the next paragraph that increasing the amount of carbon dioxide by adding the gas is not effective in intensifying the depression invalidates the suggested explanation.

Analyses were also made of the amount of carbon dioxide gas required to produce a certain pH. It was found that more of the gas is present in the water at a given pH than is set free in the water by adding other acids to the same pH. This result would be expected, since it is highly probable that when the water is acidified, considerable time must elapse before all of the acid reacts with the carbonate of the water. Consequently immediately after acidification part of the free hydrogen ions present are derived from the acid added, and not from the reaction of carbon dioxide with water. The difference in carbon dioxide content between water acidified with carbon dioxide gas and water acidified by other acids is small at pH 7.0 or even 6.5 but at pH 6.0 there is three times and at pH 5.0 nearly ten times as much carbon dioxide gas in the water as is liberated by mineral acids at those same hydrogen ion concentrations. These results are shown in Fig. 2.

We are thus faced with the question—why is carbon dioxide not more effective as a depressing agent than any other acid, particularly at the higher acidities? There was to be sure evidence that carbon dioxide at pH 5.0 for instance has a greater depressing action than any other acid tried. Yet the difference between it and other acids is not as great as might be expected. It becomes necessary to assume, in view of the facts at hand, that the amount of depression of the respiratory rate which can

be induced in Planaria by carbon dioxide is limited and does not exceed 50 per cent. as long as the oxygen supply remains ample. If the oxygen supply is reduced, although not to a point where the respiration rate would be affected under ordinary conditions,

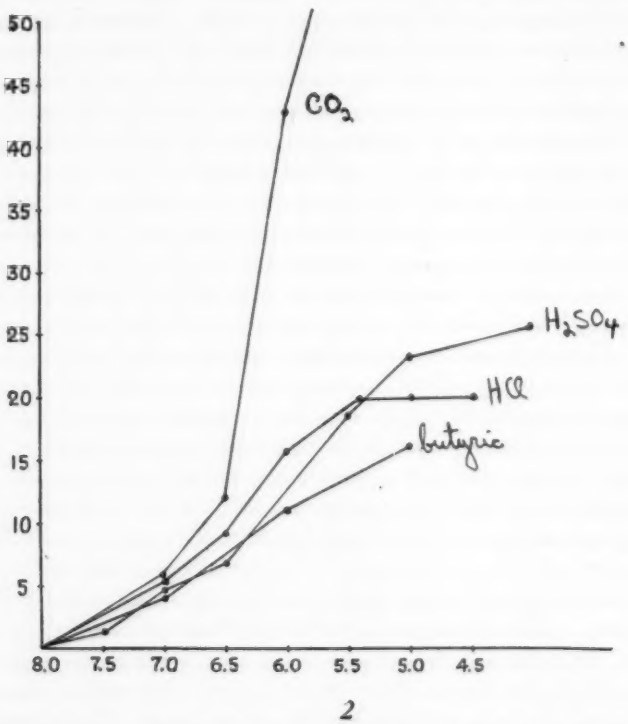

FIG. 2. Graph of the amount of carbon dioxide liberated in the well water by various acids. Amount of carbon dioxide in cc. per liter on the ordinate, pH on the abscissa. The amount of carbon dioxide gas required to produce a given pH is also shown.

the oxygen consumption can be nearly abolished if the water is saturated or nearly so with carbon dioxide. A carbon dioxide content of 30 cc. per liter appears to yield the maximum depressing effect, about 50 per cent., that can be obtained with carbon dioxide, when the oxygen supply is adequate.

6. *Conclusions Concerning the Experiments with Carbonate-free Water.*—(*a*) The depressing effect of acids on the oxygen consumption of Planaria is almost wholly abolished when carbonate-free water is used, except when the acidity is produced by carbon dioxide gas.

(*b*) An acidity due to carbon dioxide gas is equally depressing in ordinary and in carbonate-free water.

(*c*) From the statements in (*a*) and (*b*) it is concluded that the depressing action of acids on oxygen consumption is almost wholly due to the carbon dioxide liberated on acidification of natural waters.

(*d*) Different acids at the same hydrogen ion concentration do not immediately liberate the same amounts of carbon dioxide from the natural carbonate-containing water. This accounts in large part for the fact that the depressing action of different acids is not the same in degree at the same pH.

(*e*) All of the carbonate of the water used is decomposed at a pH of 4.0 to 5.0, giving a carbon dioxide content of about 3 per cent.

(*f*) The lack of action of butyric acid is not explained, for this acid also liberates carbon dioxide from the carbonates of the water. Possibly butyric acid in some way prevents the penetration of carbon dioxide into the animals.

(*g*) The amount of carbon dioxide gas required to produce a given pH is considerably greater at acidities of more than pH 6.5 than is the amount of carbon dioxide liberated in the water by other acids at the same pH. One would therefore expect that CO_2 would be much more effective as a depressing agent at acidities from 6.5 to 5.0 than any other acids. This was not noticeably the case. The difficulty could be explained by assuming that the depressing action of CO_2 reaches a maximum at a concentration of 3 per cent. and is not altered by further increase in the concentration of the gas.

(*h*) Since the depressing effect of acids is due largely to carbon dioxide and since this gas is an end product of metabolism, it becomes readily understood that organisms or parts must be affected by acids in relation to their respiratory metabolism. The higher the rate of the latter, the greater would be the depression induced by acids.

Relative Toxicity of Different Acids.

An attempt was made to discover the hydrogen ion concentration at which each of the acids used would cause the death of *Planaria* within a relatively short period of time, say, two to three hours. A sort of trial and error procedure is necessary to determine this matter. The results are not very exact but the relative toxicities of the different acids were plain enough after a few trials. Butyric acid is by far the most toxic, as found also by other investigators, and kills the animals within two or three hours at pH 5.0. Acetic acid ranks next in toxicity, killing in the time specified at pH 4.4. Tartaric and citric acids come next, killing within two or three hours at 3.6 and 3.4 respectively. The three mineral acids are nearly equally toxic, sulphuric being slightly more effective, killing in two or three hours at pH 3.2 to 3.4, while hydrochloric and nitric acids must be used at pH 3.0 to 3.2 to obtain the same result. The order of toxicity may then be expressed as follows: butyric > acetic > tartaric > citric > sulphuric > nitric = hydrochloric.

Carbon dioxide at saturation (about pH 5.2) was not found to kill the animals as long as the oxygen supply was adequate.

The death of the animals took place with equal rapidity and at the same hydrogen ion concentrations in both normal and carbonate-free water. This proves that the death is not due to the carbon dioxide set free by acids in normal water but is a direct acid effect. This is further evidenced by differences in the appearance and in the manner of disintegration of the dead animals. Death in acids appears to be generally due to coagulation. This was also noted by Mrs. Lewis, '23.

In view of the fact that the death of the animals results from a direct coagulating action of the acids while the depression of the respiratory rate by acids is caused almost wholly by carbon dioxide, no relation would be expected between the toxicity of different acids and their depressing action. This is the case. Butyric acid, the most toxic, is in non-lethal doses the poorest depressant. The experiments do not of course serve to show the amount of depression at or near the death point, except in the cases of acetic and butyric acids, pH 5.0, Table VI. Since different acids are equally lethal at different hydrogen ion

concentrations, it follows that the free hydrogen ions cannot be the chief factor in toxicity. The penetrating powers of the acid are probably of great importance and the nature of the anion or molecule may also be involved. Butyric and acetic acids which probably penetrate organisms the most readily of all the acids employed are also the most toxic. In a study of the toxicity of various acids for ciliate Protozoa, Collett ('19) reached the conclusion that hydrogen ion concentration is not the most important factor.

Summary.

1. The effect of acidification of the medium on the rate of oxygen consumption of aquatic organisms was studied.

2. The acids used were: hydrochloric, nitric, sulphuric, carbonic, butyric, acetic, citric, and tartaric. They were added to water to produce acidities ranging from pH 7.5 to 5.0, at intervals of 0.5 pH.

3. *Planaria dorotocephala* was the chief animal used as material. Some tests with hydrochloric acid were also made using starfish and nudibranchs.

4. The acidification of natural waters, either salt or fresh, (pH 8.0), by any of the acids used except butyric causes a decrease in the rate of oxygen consumption at all acidities greater than pH 7.0. The majority of acids also cause depression between 7.8 and 7.0 but in the case of acetic acid and more doubtfully carbonic, there was some tendency towards a slight acceleration of the rate of oxygen consumption at these lower concentrations.

5. The decrease in the rate of oxygen consumption due to acids is completely and promptly reversible, as long as the animals are not actually injured.

6. The acidification of fresh water from which all carbonates have been previously removed has no or only a slight effect upon the rate of oxygen consumption of *Planaria*, except when the acidity is produced by carbon dioxide.

7. The depressing action of carbon dioxide is the same whether the gas is added to ordinary or to carbonate-free water.

8. From 6 and 7 it follows that the depressing action of acids in natural waters is due chiefly or wholly to the carbon dioxide which they liberate from the carbonates of such waters.

9. The depressing action of acidified natural waters on the rate of oxygen consumption of *Planaria* is not the same with different acids at the same hydrogen ion concentration. This appears to be due largely to the fact that the amount of carbon dioxide immediately liberated from the carbonates of the water differs with different acids at the same pH.

10. The depressing action of acids in natural waters is greater the greater the acidity up to an acidity of about pH 5.0. This is due to the fact that the more acid added, the greater is the quantity of carbon dioxide liberated.

11. Lower concentrations are, however, relatively more effective than higher ones.

12. The maximum amount of depression of oxygen consumption that can be induced by acids is about 50 per cent. This occurs at pH 4.0 to 5.0 and further acidification of the water does not increase the percentage of depression. At the acidity at which the maximum depression appears, the carbon dioxide content is about 3 per cent.

13. All of the carbonates of the fresh water employed are decomposed by acids at a pH of 4.0 to 5.0, producing a carbon dioxide concentration of 3 per cent. This might explain the facts given in 12 were it not that concentrations of carbon dioxide gas much higher than 3 per cent. do not increase the percentage of depression beyond 50 per cent., as long as the oxygen supply is ample. Concentrations of carbon dioxide gas up to 25 per cent. were tested.

14. From the facts cited in 13 it appears necessary to assume that the depression of the rate of oxygen consumption which can be induced by carbon dioxide does not exceed 50 per cent. as long as the oxygen supply is adequate.

15. A combination of high carbon dioxide content and low oxygen content practically abolishes the oxygen consumption of *Planaria*, even though the oxygen content used (2 cc. per liter) would be ample for normal respiration in the absence of carbon dioxide.

16. No explanation has been discovered for the differences between the action of carbon dioxide and other acids at low concentrations (pH 7.5 and 7.0).

17. Butyric acid has almost no action on the oxygen consumption of *Planaria*. No explanation has been discovered for this fact.

18. The different acids employed are lethal for *Planaria* in a given arbitrarily selected time (2 to 3 hours) at different hydrogen ion concentrations. The order of toxicity and the hydrogen ion concentrations at which the acids are equally lethal are: butyric (5.0), acetic (4.4), tartaric (3.6), citric (3.4), sulphuric (3.2) and nitric and hydrochloric (3.0).

19. The facts given in 18 prove that the hydrogen ion is not the cause of death but either the anion or the molecule of the acid is involved. Penetrability is probably also a factor. Death in acids appears to be due to coagulation.

20. The order of toxicity of the acids and the pH at which they are equally lethal are the same in ordinary and in carbonate-free water, showing that death is not due to carbon dioxide liberated.

21. Acidification of natural waters constitutes a method for depressing the rate of oxygen consumption of aquatic animals for experimental purposes.

22. The experiments herein presented cast doubt on the supposed importance of hydrogen ion concentration *per se* in biological processes.

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